THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DAVID W. BISHOP, Carnegie Institution of Washington
FRANK A. BROWN, JR., Northwestern University
JAMES CASE, State University of Iowa
JOHN W. GOWEN, Iowa State College
LIBBIE H. HYMAN, American Museum of Natural History
J. LOGAN IRVIN, University of North Carolina
JOHN H. LOCHHEAD, University of Vermont
V. L. LOOSANOFF, U. S. Fish and Wildlife Service
C. L. PROSSER, University of Illinois
BERTA SCHARRER, Albert Einstein College of Medicine
FRANZ SCHRADER, Duke University
WM. RANDOLPH TAYLOR, University of Michigan
DONALD P. COSTELLO, University of North Carolina
Managing Editor

VOLUME 120
JANUARY TO JUNE, 1961

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1. February, 1961

PAGE

Chiakulas, John J., and Lawrence E. Scheving
The cyclic nature and magnitude of cell division in gastric mucosa of urodele larvae reared in the pond and laboratory 1

Crouse, Helen V.
X-ray effects on sex of progeny in Sciara coprophila 8

Harvey, William R.
The respiratory exchange of diapausing pupae of the cecropia silkworm in the presence of carbon^{14} monoxide 11

Humphries, A. A., Jr.
Experiments on the normal meiotic block in the ovum of Triturus viridescens 29

Lagerspetz, Kari, and Aila Lehtonen
Humidity reactions of some aquatic isopods in the air 38

Lagerspetz, Kari, and Maija Mattila
Salinity reactions of some fresh- and brackish-water crustaceans 44

Lynn, W. Gardner, and James Norman Dent
A comparison of the responses of Triturus and Desmognathus to thyroid-stimulating hormone administration 54

Miller, Milton A., and W. D. Burbanck
Systematics and distribution of an estuarine isopod crustacean, Cyathura polita (Stimpson, 1855), new comb., from the Gulf and Atlantic seaboard of the United States 62

Ritchie, Don
Effects of cultural variation on cell size in a marine fungus 85

Scheltema, Rudolf S.
Metamorphosis of the veliger larvae of Nassarius obsoletus (Gastropoda) in response to bottom sediment 92

Schiffman, R. H.
A perfusion study of the movement of strontium across the gills of rainbow trout (Salmo gairdnerii) 110

Tweedell, Kenyon S.
Regeneration of the enteropneust, Saccoglossus kowalevskii 118

No. 2. April, 1961

Augenfeld, John M., and John C. Neess
Observations on the respiratory enzymes of various life-stages of Chironomus plumosus, Chironomus staegeri, and Aedes aegypti 129

ENGELS, WILLIAM L.
Photoperiodism and the annual testicular cycle of the bobolink (Dolichonyx oryzivorus), a transequatorial migrant, as compared with two Temperate Zone migrants 140
ENRIGHT, J. T.
Lunar orientation of Orchestoidea corniculata Stout (Amphipoda)..... 148
JACOBS, M. E.
Sex differences and environmental influence on dopa-oxidizing activity in Drosophila melanogaster..... 157
NORRIS, KENNETH S., JOHN H. PRESCOTT, PAUL V. ASA-DORIAN AND PAUL PERKINS
An experimental demonstration of echo-location behavior in the porpoise, Tursiops truncatus (Montagu)..... 163
OKAZAKI, KAYO
Skeleton formation of sea urchin larvae. III. Similarity of effect of low calcium and high magnesium on spicule formation..... 177
ROSENTHAL, HAROLD L.
The uptake and turnover of S^{35} sulfate by Lebistes..... 183
SANDEEN, MURIEL I., AND JOHN D. COSTLOW, JR.
The presence of decapod-pigment-activating substances in the central nervous system of representative Cirripedia..... 192
SHEPHARD, DAVID C.
A cytological study of the origin of melanophores in the teleosts..... 206
STUNKARD, HORACE W.
Cercaria diptocerca Miller and Northrup, 1926 and Stephanostomum dentatum (Linton, 1900) Manter, 1931..... 221
THORSON, THOMAS B.
The partitioning of body water in Osteichthyes: Phylogenetic and ecological implications in aquatic vertebrates..... 238
UKELES, RAVENNA
The effect of temperature on the growth and survival of several marine algal species..... 255
WELLS, HARRY W., MARY JANE WELLS AND I. E. GRAY
Food of the sea-star Astropecten articulatus..... 265
YANAGIMACHI, RYUZO
Studies on the sexual organization of the Rhizocephala. III. The mode of sex-determination in Peltogasterella..... 272

No. 3. JUNE, 1961

COSTELLO, DONALD P.
On the orientation of centrioles in dividing cells, and its significance: A new contribution to spindle mechanics..... 285
COWDEN, RONALD R.
A cytochemical investigation of oögenesis and development to the swimming larval stage in the chiton, Chiton tuberculatum..... 313
ELLS, H. A., AND C. P. READ
Physiology of the vinegar eel, Turbatrix aceti (Nematoda). I. Observations on respiratory metabolism..... 326

FINGERMAN, MILTON, R. NAGABHUSHANAM AND LORALEE PHILPOTT
Physiology of the melanophores in the crab Sesarma reticulatum..... 337
DE GENNARO, LOUIS D.
The carbohydrate composition of the glycogen body of the chick embryo as revealed by paper chromatography..... 348
GRAY, I. E.
Changes in abundance of the commensal crabs of Chaetopterus..... 353
HATHAWAY, RALPH R., AND CHARLES B. METZ
Interactions between Arbacia sperm and S^{35}-labelled fertilizin..... 360
JENKINS, MARIE M.
Respiration rates in planarians. II. The effect of goitrogens on oxygen consumption..... 370
RUCK, PHILIP
Photoreceptor cell response and flicker fusion frequency in the compound eye of the fly, Lucilia sericata (Meigen)..... 375
SCHUEL, HERBERT
A study of the effects of urethan on the cleavage of the Chaetopterus egg. I. Inhibition of cleavage..... 384
SINDERMANN, CARL J., AND DONALD F. MAIRS
A blood group system for spiny dogfish, Squalus acanthias L..... 401
STEINBACH, H. B., AND PHILIP B. DUNHAM
Ionic gradients in some invertebrate spermatozoa..... 411
SWAN, EMERY F.
Some observations on the growth rate of sea urchins in the genus Strongylocentrotus..... 420

Volume 120 Number 1

QH 301 B59 v.120 no 1

THE UNIVERSITY OF MICHIGAN SCIENCE LIBRARY

7R

THE BIOLOGICAL BULLETIN

PUBLISHED BY

THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DAVID W. BISHOP, Carnegie Institution of Washington
FRANK A. BROWN, JR., Northwestern University
JAMES CASE, State University of Iowa
JOHN W. GOWEN, Iowa State College
LIBBIE H. HYMAN, American Museum of Natural History
J. LOGAN IRVIN, University of North Carolina
JOHN H. LOCHHEAD, University of Vermont
V. L. LOOSANOFF, U. S. Fish and Wildlife Service
C. L. PROSSER, University of Illinois
BERTA SCHARRER, Albert Einstein College of Medicine
FRANZ SCHRADER, Duke University
WM. RANDOLPH TAYLOR, University of Michigan
DONALD P. COSTELLO, University of North Carolina
Managing Editor

FEBRUARY, 1961

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A ***condensed title*** or running head of no more than 35 letters and spaces should be included.

4. ***Literature Cited.*** The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. ***Figures.*** The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction, but not larger than 15 × 22 inches. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. ***Mailing.*** Manuscripts should be packed flat. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Copyright © 1961, by the Marine Biological Laboratory

Second-class postage paid at Lancaster, Pa.

Vol. 120, No. 1 February, 1961

17
173
884/A

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE CYCLIC NATURE AND MAGNITUDE OF CELL DIVISION IN GASTRIC MUCOSA OF URODELE LARVAE REARED IN THE POND AND LABORATORY

JOHN J. CHIAKULAS AND LAWRENCE E. SCHEVING

Department of Anatomy, The Chicago Medical School, Chicago 12, Illinois

In a previous publication, Scheving, Chiakulas and Abzug (1959) reported that in salamander larvae the epidermal mitotic rate in animals from two different environments was characteristically cyclic over a 24-hour period. Although the cycle was manifest and corresponded as to time in both groups, the over-all mean mitotic rate of the epidermis in animals collected from the natural pond environment was 20 times that in the epidermis of laboratory-reared animals. A number of differences in the ecological conditions of the pond and laboratory environments were presented as possible factors responsible for the differential in the mitotic rates of the two groups. The results of our study on epidermis bring up a number of questions: (1) Do these ecological factors, possibly responsible for the differences in the mitotic rates, act specifically on the epidermis alone, since this tissue is directly exposed to the external environment, or do they act in an unspecific indirect manner so as to affect the general physiology of the animal and thereby affect the mitotic rate of other tissues as well? (2) Is the 24-hour cycle, characteristic of the mitotic rate of epidermis, present in other tissues?

To furnish possible answers to these questions, it was decided to study the magnitude and cyclic nature of the mitotic rate of the gastric mucosal epithelium of the same two groups of animals, pond and laboratory, used previously for the epidermal mitotic rate determinations. If a difference existed between the magnitude of mitosis in the two groups, a general physiological effect of environmental factors might be deduced. Other investigators (Klein and Geisel, 1947; Leblond and Stevens, 1948) have indicated that gut mucosal epithelium in the rat shows no significant cyclic variations in mitotic rate but that this tissue has a constant renewal rate. Therefore, it was of interest to determine if the phenomenon of daily cyclic variation in the mitotic rate of gastric mucosal epithelium existed in salamander larvae, and if so, what similarities or differences existed between the cycles of gastric mucosal epithelium and epidermis. The results of the determinations on the mitotic rate of gastric mucosal epithelium are the basis of this report.

Copyright © 1961, by the Marine Biological Laboratory

Materials and Methods

Two groups of *Amblystoma punctatum* larvae, which were used in an earlier study of the epidermal mitotic rate (Scheving *et al.*, 1959), were used in the present study. The two groups were: (1) "laboratory" animals, which were reared in the laboratory from the egg stage until the chronological age of 6 weeks after hatching, and (2) "pond" animals, which were collected as larvae from the pond and which were approximately at the same stage of development as the laboratory animals when sacrificed. The differences in the two environments cannot be rigidly defined since no control procedures were used. It may be stated, however, that the laboratory animals were reared in city tap water which was run through commercial filters to remove the chlorine. In contrast, the pond animals grew in the semi-stagnant water of the natural pond. During the 24-hour collection period, water temperatures were taken hourly, and the temperature of the pond water ranged between 16° C. and 21° C. The laboratory animals were raised

Table I

Mean mitotic indices (mitoses/1000 cells) with standard deviations of gastric mucosal epithelium

Hour	Laboratory animals	Pond animals
6:30 A.M.	0.11 ± 0.07	2.89 ± 0.44
8:30 A.M.	0.33 ± 0.12	2.89 ± 0.69
10:30 A.M.	1.21 ± 0.40	5.65 ± 0.80
12:30 P.M.	1.09 ± 0.66	6.01 ± 1.62
2:30 P.M.	0.88 ± 0.35	4.60 ± 0.61
4:30 P.M.	0.97 ± 0.34	4.32 ± 1.11
6:30 P.M.	0.38 ± 0.40	4.14 ± 0.81
8:30 P.M.	0.31 ± 0.13	4.40 ± 0.85
10:30 P.M.	0.35 ± 1.14	4.60 ± 1.42
12:30 A.M.	0.41 ± 0.15	5.24 ± 0.92
2:30 A.M.	0.50 ± 0.12	7.02 ± 1.23
4:30 A.M.	0.43 ± 0.18	3.90 ± 0.63
Over-all Daily Mean	0.58 ± 0.34	4.64 ± 0.93

from the egg stage at heated room temperature, ranging from 20° C. to 24° C. During the 24-hour period of sacrifice, the room temperature averaged 21° C. Other possible differences between the two environments, such as differences in the oxygen tension and salt concentration of the water, degree of activity of the animals, were not determined.

Groups of 20 animals from each environment were sacrificed every two hours over a 24-hour period, fixed in Bouin's, sectioned transversely and stained with iron hematoxylin. Nuclear and mitotic counts were made on the gastric mucosal epithelium. Only the cells lining the lumen of the stomach were included in the counts; areas of continuity of the mucosal epithelium with the gastric glands were excluded. To avoid duplication, the number of nuclei and mitotic figures was recorded for every third section. At least 5000 nuclei were counted in each specimen. The mitotic index was calculated as the number of mitoses per 1000 cells. The mean mitotic indices for each time-period group and the mean over-all mitotic indices for the 24-hour periods, for both laboratory and pond animals, were determined and are indicated in Table I. Statistical comparisons and analyses were

made by computing the standard error of the differences between two means and the consequent probability.

Results

The mean mitotic indices of the gastric mucosal epithelium for each of the 12 time periods over a 24-hour day and for both animal groups are listed in Table I. When these indices are plotted in a graph (Figs. 1, 2), it is obvious that the mucosal mitotic activity of both the laboratory and pond animals is characteristically cyclic in nature.

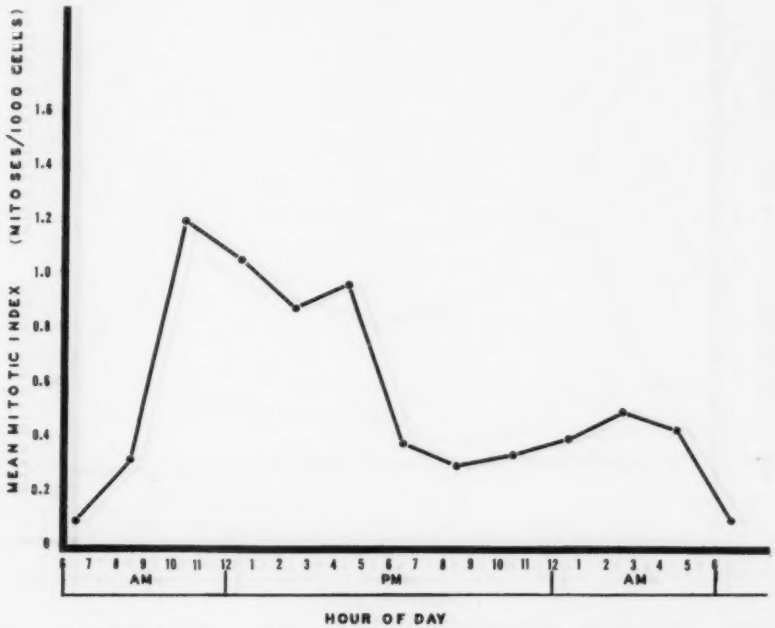


Figure 1. Mean indices, at two-hour intervals over a 24-hour period, for mitotic rate of gastric mucosal epithelium of laboratory animals.

In the laboratory animals the peak hour of activity is at 10:30 A.M., at which time the mean mitotic index is 1.21 ± 0.40 (Table I; Fig. 1). Following this peak, a gradual decrease in the number of dividing cells is recorded until 4:30 P.M., when the daily over-all mean mitotic index of 0.58 ± 0.34 is approached. Subsequently, there is a relatively rapid decrease in mitotic activity and the mitotic indices are below the 24-hour mean index during a seemingly stable period from 6:30 P.M. through 6:30 A.M., with the exception of a minor increase at 2:30 A.M.

The mitotic index of the 10:30 A.M. peak was compared with the over-all mean mitotic rate and the difference was found to be statistically significant ($0.001 < p < 0.01$). When the 10:30 A.M. peak index was compared to the 6:30 A.M.

minimum index, the difference indicated greater statistical significance ($p < 0.001$). It also can be seen from Table I and Figure 1 that the mitotic rate at 10:30 A.M. is twice the average rate for all hours and 10 times the minimum rate recorded at 6:30 A.M. The minor rise in the rate of cellular division observed at 2:30 A.M. has no statistical significance.

In the pond animals, two peaks of high cellular division occur—one at 12:30 P.M. when the mean mitotic index reaches the value of 6.01 ± 1.62, and another at 2:30 A.M. when the mean mitotic index of 7.02 ± 1.23 is recorded (Table I; Fig. 2). When these peak values are compared to the daily over-all mean mitotic

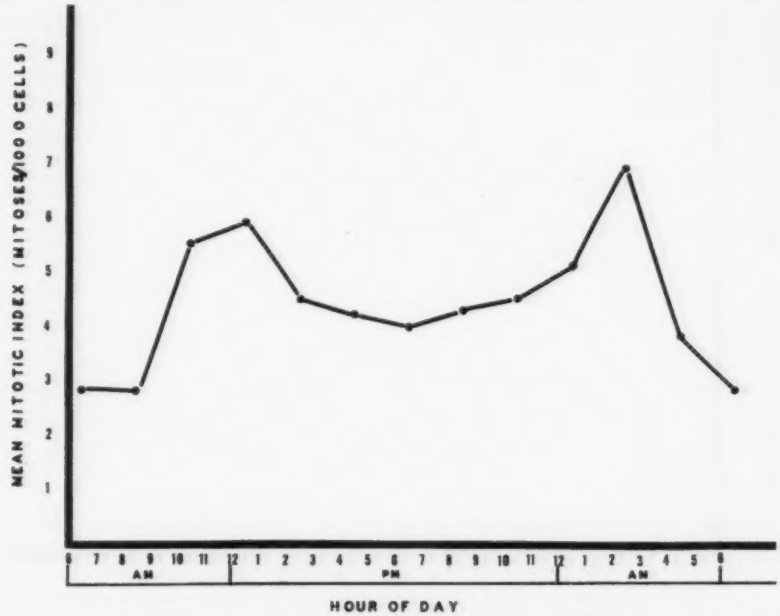


FIGURE 2. Mean indices, at two-hour intervals over a 24-hour period, for mitotic rate of gastric mucosal epithelium of pond animals.

index of 4.64 ± 0.93, they are not statistically significant. However, when these two values are compared to the minimum index of 2.89 ± 0.44 occurring at 6:30 A.M., both the differences are statistically significant ($p < 0.01$).

When the mitotic indices of both laboratory and pond animals are plotted on the same scale (Fig. 3) a number of interesting observations are made. It is evident from the graphs (Fig. 3) that the rate of cell division follows a similar cyclic pattern in both groups, with a high period of activity between 10:30 A.M. and 12:30 P.M. The 2:30 A.M. peak present in the pond group is not present in the laboratory group, although it is probable that the slight rise at 2:30 A.M. above the preceding period may represent a rather feeble acceleration corresponding to the peak present at this time in the pond group. Minimum mitotic activity

is established in both environmental groups between the hours of 6:30 A.M. and 8:30 A.M.

The most striking difference in the two animal groups is in the magnitude of the mitotic rates. The over-all mean mitotic rate in the gastric mucosa of the pond animals is approximately 8 times that of the laboratory animals (see Table I; compare two graphs of Figure 3).

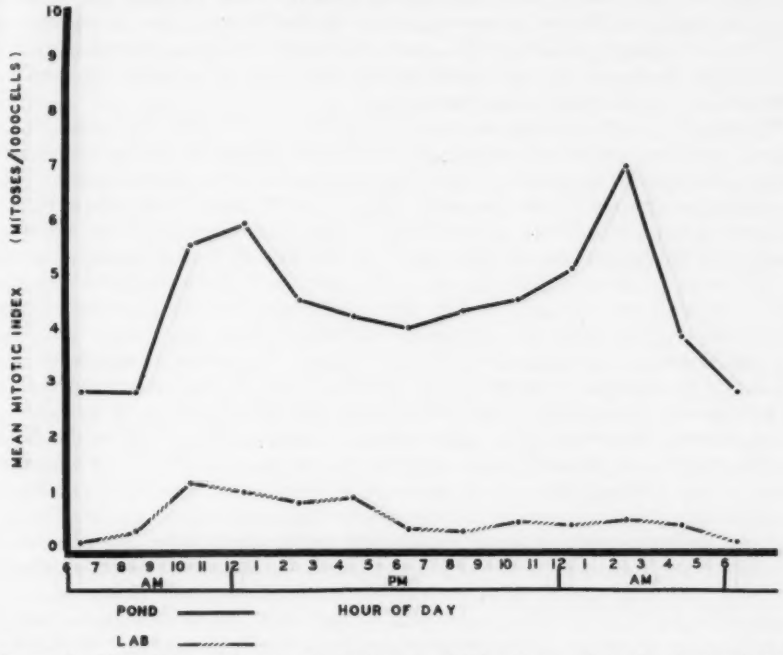


FIGURE 3. Mean indices of mucosal mitotic rate of laboratory and pond animals plotted on the same scale.

DISCUSSION

It is not the intent of this report to review the rapidly accumulating literature dealing with the cyclic nature of the rate of mitosis in various tissues or on the periodicity observed in many other biological processes. Brief summaries or reviews of some of the pertinent investigations in this field have been made by Scheving (1959), Leedale (1959) and Harker (1958). Here, we will discuss only the significance and implications of our results and relate and compare these results to those obtained in a similar investigation of the mitotic rate in urodele larval epidermis (Scheving *et al.*, 1959).

The results of this investigation, first of all, clearly demonstrate that the mitotic rate of the gastric mucosal epithelium is cyclic in character with occurrence of typical peaks and lows in the rate of cellular division during the 24-hour period.

Secondly, this finding indicates that gut mucosa is not a tissue with a constant replacement rate, at least in urodele larvae, and therefore this finding differs from the conclusions of Klein and Geisel (1947), and Leblond and Stevens (1948) that gut mucosal epithelium in the rat demonstrated no significant periodic fluctuations in its mitotic rate. Although a great number of previous investigations have dealt with periodicity of mitotic rate of epidermis or cornea, our results add to the evidence that other tissues may have similar cycles. What the basic factor underlying the cause of this cyclic nature of the cellular division rate is remains unanswered. However, whatever the cause, whether it be an intrinsic characteristic of the cells themselves or an externally imposed factor, it is apparent that it is operative in several tissues simultaneously.

When the graphs of Figure 3 are compared, it is noted that although the mucosal mitotic rate of both pond and laboratory animals is cyclic, the over-all daily mitotic rate of the pond group is 8 times that of the laboratory animals' rate. A similar but greater differential was reported for the epidermal mitotic rates of the same animals (Scheving *et al.*, 1959). Again, the reason for this difference cannot be explained at the present time. In the report on epidermis, a number of differences in the ecological factors of the pond and laboratory environments—such as oxygen tension, activity, diet, salt concentration—were mentioned as possible causes for the differences in mitotic rates between the two groups of animals. The implication was that these factors acted directly on the epidermis, since it was exposed to the external environment, or acted in some way to increase the general growth rate of the animals. Since this differential in mitotic rate is present also in the mucosal epithelium of the same animals, it appears that whatever the factors are that operate in producing this increased mitotic rate in pond over laboratory animals, they act in general to result in an enhanced rate of growth. However, this inference does not preclude the possibility that some of the ecological factors may still be acting directly on the epidermal tissue, because the gastric mucosal rate of the pond animals is only 8 times that of the laboratory animals, whereas the pond epidermal rate is 20 times that of the laboratory rate. Also, when it is considered that for the 24-hour period the mean mucosal mitotic index and the epidermal mitotic index of the laboratory animals are approximately the same, it is seen that the epidermis of the pond animals responds to a greater degree than does the mucosal epithelium. This is so probably because the epidermis is directly exposed to the environmental factors. In contrast, the mucosal epithelium, which is not directly exposed to the external environment, shows less of a mitotic response.

No correlation could be seen between the water temperature and the actual mitotic rate for specific hours. In the pond, the high temperature of 21° C. was recorded between 12:00 noon and 1:00 P.M. Correspondingly, a peak with an index of 6.01 ± 1.62 was seen in the mitotic cycle at 12:30 P.M. However, the highest peak in the cycle, with an index of 7.02 ± 1.23, occurred at 2:30 P.M. when the pond water temperature was 16.5° C. The cyclic nature of the mitotic rate thus appears to be unaffected by fluctuations in temperature over the 24-hour period. However, the mean mitotic rate over the 24 hours may be affected by the average temperature. The laboratory animals were raised at room temperature averaging 22° C. In contrast, the pond animals were subjected to colder temperatures in the pond during their period of growth, and on the day of collec-

tion the average pond temperature was only 18.5° C. Although pond and laboratory temperatures overlapped during some hours of the period over which the animals were sacrificed, at no time did the mitotic rates of the two groups show any overlap in magnitude.

The results of this investigation again point to the importance of knowing the 24-hour cyclic variations in the mitotic rates of tissues. When mitotic rates are used to interpret the results of experimental procedures, it is necessary to know if the animals involved were sacrificed at the same hours. Otherwise, comparisons and correlations or conclusions based on mitotic indices may be invalid. It is also of importance to know the conditions under which animals are reared, since differences in the environmental factors may cause great differences in the over-all mitotic rate of the tissues. Although in this investigation determinations were not made to establish the actual degree of difference between the pond and laboratory environments in terms of oxygen tension and salt concentration of the water, temperature, diet and animal activity, these differences may be responsible for the differentials seen between the mitotic rates of the two groups of animals. Controlled experiments are in progress to test the effect of each individual factor on mitotic rate of tissues.

Summary

1. The mitotic rate of gastric mucosal epithelium in both pond- and laboratory-reared urodele larvae is cyclic over a 24-hour period. Maximum rate of cell division occurs in both groups between the hours of 10:30 A.M. and 12:30 P.M.; minimum mitotic activity is recorded between 6:30 A.M. and 8:30 A.M.

2. In pond animals, the mitotic rate of gastric mucosa is 8 times the rate of the mucosa of laboratory-reared animals. The environmental differences between pond and laboratory are suggested as the underlying cause for the differential in the rates of mitosis in the two groups of animals. Since a similar difference was previously found in the epidermal mitotic rates of the same animals, it is suggested that in the pond environment an enhanced rate of growth is present.

3. The difference between the pond and laboratory mucosal daily mean mitotic rate is of lesser magnitude than the difference between the epidermal rates of the same animals. This fact indicates that the epidermis may be responding directly to exogenous environmental factors, whereas the mucosa responds only to a lesser degree since it is not directly exposed to the environmental factors.

LITERATURE CITED

Harker, J. E., 1958. Diurnal rhythms in the animal kingdom. *Biol. Rev.*, **33**: 1–52.

Klein, H., and H. Geisel, 1947. Zum Nachweis eines 24-Stundenrhythmus der Mitosen bei Ratte und Maus. *Klin. Wschr.*, **25**: 662–663.

Leblond, C. P., and C. E. Stevens, 1948. The constant renewal of the intestinal epithelium in the albino rat. *Anat. Rec.*, **100**: 357–377.

Leedale, G. F., 1959. Periodicity of mitosis and cell division in the Euglenineae. *Biol. Bull.*, **116**: 162–174.

Scheving, L. E., 1959. Mitotic activity in the human epidermis. *Anat. Rec.*, **135**: 7–20.

Scheving, L. E., J. J. Chiakulas and H. I. Abzug, 1959. A basic rhythmicity in the mitotic rate of urodele epidermis and the difference in mitotic rate between larvae reared in the laboratory and in a pond. *J. Cell. Comp. Physiol.*, **54**: 109–114.

X-RAY EFFECTS ON SEX OF PROGENY IN SCIARA COPROPHILA

HELEN V. CROUSE[1]

Department of Botany, Columbia University, New York 27, New York

Sciara coprophila characteristically gives "unisexual" progenies; *i.e.*, the offspring from any pair mating are practically all of the same sex, either males or females. This "sex of progeny" is determined by the sex chromosome constitution of the female (Moses and Metz, 1928). Genetically there are two kinds of sex chromosomes, designated X and X′ (prime). Females are either XX or XX′; the former are male-producers, the latter female-producers (Metz and Moses, 1928). Sciara males all have the same sex chromosome constitution, form only one type of sperm, and ordinarily exert no influence on the sex ratio of their progeny. The irradiation studies presented here reveal the conditions under which the male *can* influence the sex ratio of his progeny, and thereby they clarify the relationship between "sex of progeny" and the determination of sex of the individual.

All Sciara zygotes begin development with the same chromosome complement. This complement ordinarily includes three sex chromosomes, two of which are identical sister halves of the X derived from the sperm; the third one is an X or X′ derived from the egg. At the seventh or eighth cleavage an elimination of sex chromosomes occurs in the somatic nuclei: in the production of female embryos one paternally-derived X is eliminated, in the production of male embryos both are eliminated; thus the female somatic complement comes to include two sex chromosomes, the male somatic complement only one. In the embryonic germ line the three sex chromosomes are retained until after the germ cells have migrated to the definitive gonad site; then, in both sexes alike, a paternal X is eliminated, leaving two sex chromosomes in the differentiated germ line. This pattern of sex chromosome behavior indicates that sex of the individual fly is not determined prior to chromosome elimination.

The strain of flies used in the experiments reported here produced an exceedingly low number of exceptional males (males among the progeny of female-producers) and exceptional females (females among the progeny of male-producers); progeny counts on one hundred normal females bred to normal males yielded only 0.1% exceptional sex types. A number of conditions are known which may increase the percentage of exceptional sexes. An analysis of these cases (Metz and Schmuck, 1931; Reynolds, 1938; Crouse, 1960) shows the following to be most likely: (1) that the XX-X′ mechanism controls not the sex of the progeny *per se* but rather the type of chromosome elimination which takes place in the embryonic somatic nuclei at the seventh or eighth cleavage; (2) that sex of the individual fly is governed by the somatic complement; and (3) that sex differentiation follows the pattern set by the somatic complement even when the germ line contains an irregular number of sex chromosomes.

[1] The studies reported here were supported by grants G-6176 and G-9682 from the National Science Foundation.

In the experimental studies cited above (Reynolds, 1938; Crouse, 1960) cases of nondisjunction of sex chromosomes in the female germ line were analyzed: when the zygote received two sex chromosomes from the egg, the type of chromosome elimination in the embryonic soma was strictly in accordance with the XX-XX′ mechanism. That is to say, the exceptional sex types arose *not* because of errors in chromosome elimination but rather in accordance with the sex chromosome complement remaining in the embryonic soma after elimination.

The data presented below demonstrate that irradiation can influence the transmission of sex chromosomes through the male germ line and also through the female germ line, thereby giving rise to exceptional sex types.

Material and Results

Some of the data were obtained during the course of study of x-ray-induced sex-linked recessive lethals. Females heterozygous for the sex-linked markers Wavy (X′) and swollen (X) were bred to irradiated wild type males. Wavy and swollen are not alleles but they show practically no crossing-over (see Crouse, 1943). Among the progeny there occurred a number of exceptional sons in addition to the expected daughters. The data are presented in Table I. Thus, at dosages of 2000 r, 3000 r, and 4000 r exceptional sons were induced at the frequencies of 3.9%, 9.1%, and 7.2%, respectively. Moreover, all the exceptional males were matroclinous (swollen) for their sex-linked genes; none was patroclinous. These data are interpreted as follows: as a result of the irradiation, one sex chromosome instead of two was transmitted to the zygote through the sperm; then, following the elimination of this paternal X from the embryonic soma—as directed by the mother's genotype (XX′)—only one sex chromosome (maternal) remained and the embryo differentiated into a male.

Table I

Exceptional sons derived from W(sw) females bred to irradiated wild type males

Experiment	Dose	No. F_1 cultures	No. F_1 females	No. exc. + males	No. exc. sw males	% exc. sw males
M3X2	2000 r	9	337	0	14	$\frac{14}{351} = 3.9$
M3X3	3000 r	17	327	0	33	$\frac{33}{360} = 9.1$
M3X4	4000 r	22	593	0	46	$\frac{46}{639} = 7.2$

To establish with greater certainty that the exceptional males recorded in Table I arose not through x-ray-induced errors in chromosome elimination during embryogeny but rather through irregular sex chromosome transmission through the sperm, wild type males were x-rayed at 4000 r and bred to male-producing females. The following results were obtained: twelve F_1 cultures yielded a total of 234 sons and no exceptional daughters. Thus, the irradiation did not alter the kind of chromosome elimination determined by the XX constitution of the mother.

Another x-ray experiment was performed, this time on females, with a view

to determining whether irradiation could induce irregular sex chromosome transmission through the female germ line. Newly-emerged female-producing females (oöcytes in first meiotic prophase) heterozygous for Wavy (XX′) were irradiated at 4000 r and bred singly to swollen males. Normally such matings should yield families of daughters. Instead, the progeny derived from 26 matings included 991 females, 18 exceptional swollen (patroclinous) males, zero exceptional wild type (matroclinous) males. Clearly, nondisjunction of sex chromosomes was induced by the irradiation; the patroclinous sons were derived from embryos which received no sex chromosome from the egg and which underwent the type of somatic chromosome elimination called for by the mother's genotype (XX′); thereby they became XO in constitution and consequently differentiated into males. It is interesting to note that differentiation into a male can proceed normally in Sciara under the influence of a paternal X chromosome.

Discussion

The data presented here support the view argued previously (Crouse, 1960), that the exceptional sex types which arise in unisexual families are not the result of errors in chromosome elimination in the embryonic soma; instead they arise from irregular sex chromosome transmission through the male or through the female germ line. Sex of the individual fly is controlled by the somatic complement which obtains following elimination. This point of view, of course, does not explain the condition observed in some species of Sciara where both bisexual and unisexual families occur regularly, and where individual strains exhibiting one or the other condition are interfertile (see Metz, 1938).

Summary

Among the progeny of female-producers bred to irradiated males there appear an appreciable number of exceptional sons, all of which are matroclinous for their sex-linked genes. Among the progeny of male-producers bred to irradiated males, no exceptional daughters occur. Finally, when female-producers are irradiated and bred to stock males, exceptional sons appear in the progeny and all of them are patroclinous for their sex-linked genes. These observations, taken collectively, are interpreted as follows: irradiation of sperm or of oöcytes can result in the transmission to the zygote of an irregular number of sex chromosomes; if, following elimination, a viable somatic complement (XX or XO) obtains, the embryo will differentiate accordingly into female or male.

LITERATURE CITED

Crouse, H. V., 1960. The nature of the influence of X-translocations on sex of progeny in *Sciara coprophila*. *Chromosoma*, **11**: 146–166.

Metz, C. W., 1938. Chromosome behavior, inheritance and sex determination in Sciara. *Amer. Nat.*, **72**: 485–520.

Metz, C. W., and M. Moses, 1928. Observations on sex-ratio determination in Sciara (Diptera). *Proc. Nat. Acad. Sci.*, **14**: 930–932.

Metz, C. W., and M. L. Schmuck, 1931. Studies on sex determination and the sex chromosome mechanism in Sciara. *Genetics*, **16**: 225–253.

Moses, M., and C. W. Metz, 1928. Evidence that the female is responsible for the sex ratio in Sciara (Diptera). *Proc. Nat. Acad. Sci.*, **14**: 928–930.

Reynolds, J. P., 1938. Sex determination in a bisexual strain of *Sciara coprophila*. *Genetics*, **23**: 203–220.

THE RESPIRATORY EXCHANGE OF DIAPAUSING PUPAE OF THE CECROPIA SILKWORM IN THE PRESENCE OF CARBON^{14} MONOXIDE [1]

WILLIAM R. HARVEY [2]

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

The inhibition of cytochrome oxidase by carbon monoxide stems from the remarkable similarity of this gas to oxygen. Recent work has shown that this similarity is even more far-reaching than formerly suspected, and that the combination of carbon monoxide with cytochrome oxidase may lead to phenomena in addition to a simple inhibition of respiration. For example, carbon monoxide may actually stimulate respiration (Daly, 1954). It may be combusted by oxygen, either enzymatically (Breckenridge, 1953), or catalytically; it may combine with water or alkali to produce formic acid or formates (Warburg, 1927).

The combustion of carbon monoxide has been studied by several workers including Clark, Stannard and Fenn (1950) and Black and Tyler (1959a, 1959b). The last-mentioned workers present a useful summary of the recent work on the oxidation of carbon monoxide, while the larger subject of the effects of carbon monoxide on living organisms has been ably reviewed by Lilienthal (1950).

Since the combustion of carbon monoxide may be superimposed on either the stimulation or the inhibition of respiration by carbon monoxide, the combustion of this gas has significant ramifications in studies of the carbon monoxide-inhibition of respiration. With present techniques for the measurement of radioactivity and for the analysis of small samples of respiratory gases, it is now feasible to disentangle the combustion of carbon monoxide from its effects on respiration.

The study reported here has combined these techniques to re-examine the respiratory exchange of diapausing pupae of the Cecropia silkworm in the presence of carbon monoxide and oxygen. A complete analysis has been achieved; namely, the simultaneous measurement of oxygen uptake, total gas uptake, and the production of $C^{14}O_2$ from $C^{14}O$. The results make possible a clear understanding of the complicated effects of carbon monoxide on the metabolism of diapausing pupae; they also provide new insight into the reaction of cytochrome oxidase with oxygen.

Materials and Methods

1. *Experimental animals*

The experiments were performed on diapausing pupae of the Cecropia silkworm which were reared under nets on wild cherry trees and managed as described

[1] This investigation was supported by a research grant E-2110 from the National Institute of Allergy and Infectious Diseases of the U. S. Public Health Service and by a grant from the Milton Fund, Harvard University.

[2] Present address: Institute of Biological Chemistry, University of Copenhagen, Copenhagen, Denmark.

by Shappirio and Williams (1957). Certain pupae were used during the five-month period of stable diapause (Williams, 1956); others were placed in permanent diapause by the removal of their brains (Williams, 1946).

In all experiments using radioactive carbon monoxide, the most anterior abdominal spiracles were cannulated with a short section of 0.011-inch bore polyethylene tubing (Clay-Adams) to eliminate the intermittent release of carbon dioxide described by Schneiderman and Williams (1955) and Buck and Keister (1955). Since the cannulation sometimes resulted in minor integumentary injury, the pupae were routinely stored at 25° C. for at least a month prior to use.

During the CO-combustion experiments the pupae were maintained in the dark with occasional exposure to dim light. Since the cuticle of the pupae is quite opaque the experiments may be viewed as having been conducted in the dark.

2. *Manometric measurements of gas uptake*

The rate of uptake of oxygen (Q_{O_2}) by pupae in air at 25° C. was determined by a modification of Warburg's "direct method" (Schneiderman and Williams, 1953). For the purpose of absorbing carbon dioxide, two filter papers moistened with a total of 1.6 ml. of 1 N potassium hydroxide were sealed in each vessel. The Q_{O_2} was expressed as the microliters of oxygen consumed per gram of initial live weight per hour. The initial live weight is the weight of the pupa within two months after pupation.

The measurements of carbon monoxide uptake reported in Section 2 of the Results and of total gas uptake in $CO\text{-}O_2$ mixtures at atmospheric pressure, reported in Sections 1 and 8 of the Results, were performed by the technique just described.

3. *Volumetric measurements of total gas uptake*

a) *At elevated pressures*

Measurements of total gas uptake at elevated pressures were performed at 25° C. in 45-ml. capillary volumeters by the use of the high pressure technique of Schneiderman and Feder (1954).

b) *At atmospheric pressure*

Except for the results reported in Sections 1 and 8, all measurements of total gas uptake in $CO\text{-}O_2$ mixtures were performed at 25° C. in simple 8- or 15-ml. capillary volumeters (Fenn, 1935) similar to the one described by Schneiderman and Feder (1954) but modified as shown in Figure 1 to permit the sampling of gases. The volumeter was assembled and flushed with 20 volumes of the experimental gas introduced through the vaccine stopper by means of a 22-gauge needle, and expelled *via* the capillary and attached rubber tubing. The experimental gas was trapped in the volumeter at atmospheric pressure by withdrawing the hypodermic needle and clamping off the rubber tubing. Finally, the index drop was tipped into the capillary and brought to rest at the desired point by removing the clamp on the rubber tubing and adjusting the gas volume with a 5-ml. syringe inserted through the vaccine stopper and filled with the experimental gas mixture.

In each experiment one volumeter served as a thermobarometer. The latter was assembled exactly as the animal-containing volumeters except that a glass "space-occupier," displacing 6.7 ml., was substituted for the pupa.

In addition to the thermobarometer, one to four animal vessels were fastened to a Lucite holder and placed in a constant temperature bath at 25.0° C. and allowed to equilibrate for one-half hour. Barium hydroxide was then injected into the sidearm *via* the vaccine stopper. Further details of procedure are included in Section 3 of the Results.

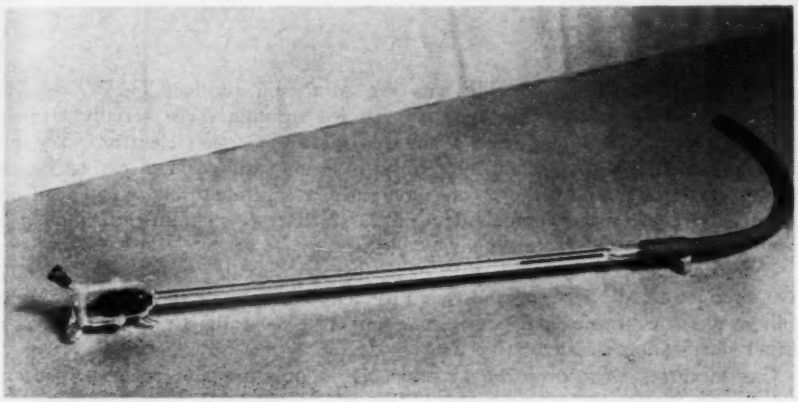


FIGURE 1. Volumeter used in the concomitant measurement at atmospheric pressure of total gas uptake, the oxygen uptake, and the production of carbon dioxide from carbon monoxide.

4. *Measurement of oxygen uptake in CO-O_2 mixtures by gas analysis*

Gas samples (ca. 1 ml.) were taken from each volumeter at the beginning and end of each experiment. The samples were analyzed for carbon dioxide and oxygen [3] in the Scholander half-cubic centimeter gas analyzer (Scholander, 1947), and the Q_{O_2} was calculated. The error introduced by the presence of carbon monoxide in the gas mixture was not in excess of 0.07% atm., as determined by analyses of carbon monoxide which had been purified as described below.

5. *Sampling and measurement of radioactive materials*

The $C^{14}O$ gas (New England Nuclear Corp.) was transferred by the method of Barton and Parrish (1956) as modified by Smetana (personal communication). Both $C^{14}O$-O_2 mixtures and $C^{14}O_2$ generated from $BaC^{14}O_3$ were counted in the gaseous phase in a Bernstein-Ballentine counting tube (Bernstein and Ballentine, 1950). The reproducibility in counting separate samples from the same gas mixture was in excess of 95%.

In certain experiments $BaC^{14}O_3$ was plated out on a millipore filter and counted as a solid on an automatic proportional flow counter (Baird Atomic; Model 750

[3] The gas analyses were performed by Mr. John Steen.

PF). When the radioactivity was less than 100 counts per minute, solid samples were counted on a windowless Geiger flow counter (Laboratory Associates, Inc.).

The volume of CO_2 that came from CO was calculated from the relationship:

$$\text{volume}_{CO_2} = \frac{\text{dpm}_{CO_2}}{\text{dpm}_{CO}} \times \text{volume}_{CO} = \frac{k\ \text{cpm}_{CO_2}}{k'\ \text{cpm}_{CO}} \times \text{volume}_{CO}$$

Provided both $C^{14}O$ and $C^{14}O_2$ are counted under identical conditions, the constants k and k′ will be equal and the volume of carbon monoxide combusted can be calculated directly.

6. *Experimental gases*

The carrier carbon monoxide was the Matheson product (C.P.) assaying 99.9% carbon monoxide. This gas was passed through three serially arranged wash bottles, each equipped with a sintered glass dispersion element. The first bottle contained 10% potassium hydroxide to remove any residual carbon dioxide and carbonyl compounds; the second contained a saturated solution of lead acetate to remove any sulfur compounds; and the third vessel contained distilled water to saturate the gas with water vapor.

U.S.P. grade oxygen and U.S.P. grade carbon dioxide, each assaying 99.5%, were obtained from New England Gas Products, Inc. Since the major impurities in these gases were nitrogen, argon, oxygen (0.08% in the CO_2), and H_2O, no further purification was made.

Gas mixtures were prepared at atmospheric pressure and room temperature in a 6-liter, water-sealed spirometer. The gas composition was determined accurately with the Scholander gas analyzer (Scholander, 1947) immediately before use.

Experimental Results

1. *Manometric evidence for the combustion of carbon monoxide*

In manometric experiments there is no quantitative way to distinguish between gas disappearance due to the combustion of carbon monoxide and that due to respiration. However, it is relatively easy to discover in a qualitative sense whether the combustion of carbon monoxide is going on. It will be recalled that this combustion involves the disappearance of one molecule of oxygen for every two molecules of carbon monoxide that are burned. A known volume of oxygen and an excess of carbon monoxide can be sealed in a Warburg flask, together with an animal and alkali. If all of the oxygen is removed by carbon monoxide-combustion, the amount of gas disappearing from the flask will be three times the known amount of oxygen present; if all of the oxygen is removed by respiration, the amount of gas disappearing will be equal to the amount of oxygen present; and finally, if part of the oxygen is removed by carbon monoxide-combustion and part by respiration, the total amount of gas disappearing will assume an intermediate value (Thimann *et al.*, 1954).

Six preparations of this type were assembled. In each preparation a diapausing pupa was sealed in a 45-ml. vessel together with a filter paper moistened with 1.6 ml. of 1 N potassium hydroxide. The vessels were each flushed with a slowly

flowing mixture of 99% atm. carbon monoxide and 1% atm. oxygen for 15 hours and then sealed. The total amount of oxygen initially present in each animal vessel was therefore 1% of the gas volume of the sealed system. A seventh air-filled vessel served as thermobarometer.

The time course of total gas uptake in a typical preparation, corrected for the reaction of carbon monoxide with alkali as described below, is plotted in Figure 2. The amount of oxygen present at the outset is indicated on the graph. It will

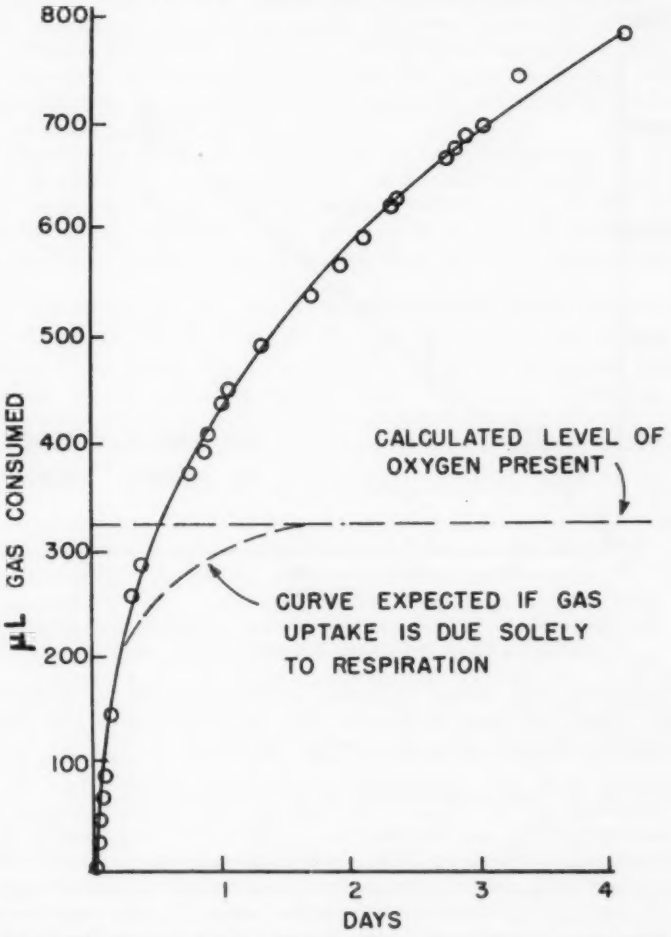


FIGURE 2. The time-course of the disappearance of gases from a sealed Warburg vessel containing a diapausing pupa in the presence of alkali and a gas mixture of 99% atm. carbon monoxide and 1% atm. oxygen. Each datum has been corrected for the reaction of carbon monoxide with the alkali.

be observed that gas continued to disappear long after the calculated 325 μl. of oxygen would have been used up. The substantial anaerobic capacity of the pupae (Harvey and Williams, 1958a; 1958b) enabled the continuation of the experiment for four days. At that time the function was tending toward a plateau somewhat short of the 975 μl. (325 μl. of oxygen plus 650 μl. of carbon monoxide) predicted for removal of the oxygen solely by carbon monoxide-oxidation. These results, together with the similar results obtained with the other five preparations,

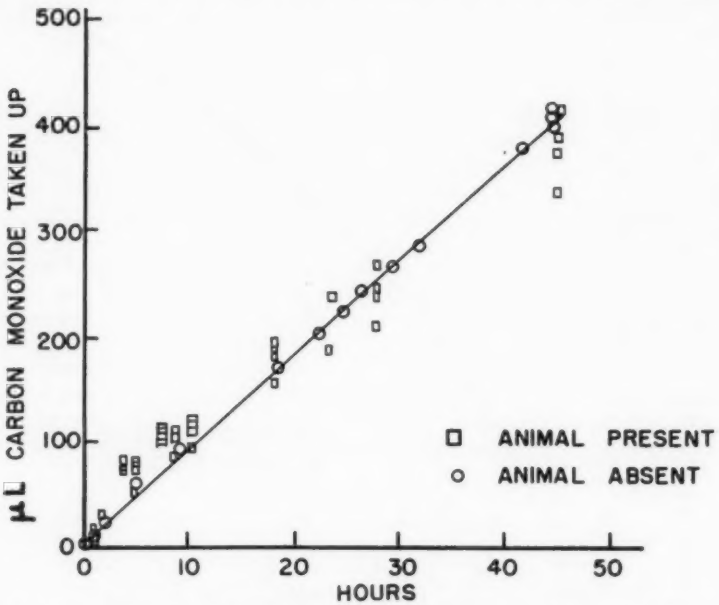


FIGURE 3. The time-course of the disappearance of carbon monoxide from Warburg vessels containing alkali and carbon monoxide, but no oxygen.

clearly demonstrate that carbon monoxide is removed from the system by the living pupa. Since this does not occur in the absence of oxygen (see Section 2) one may infer that carbon monoxide is being combusted.

2. Correction for the non-oxidative removal of carbon monoxide

The manometric data just described were corrected for the reaction of carbon monoxide with alkali (Warburg, 1927) in the following way. Four 45-ml. Warburg vessels were prepared as in the above experiment but no animals were added. Three were flushed with 20 volumes of carbon monoxide that had been washed through potassium hydroxide and lead acetate (Method 6). The fourth vessel contained air and served as a thermobarometer. The uptake of carbon monoxide from each vessel, plotted as a function of time in Figure 3, amounted to 9.2 ± 0.2 (S.D.) μl./hour.

An essentially similar experiment was performed to determine whether the living pupa catalyzed any further non-oxidative uptake of carbon monoxide. When a pupa was added to the system the rate of carbon monoxide uptake was identical with that in the absence of the pupa (Fig. 3). Therefore, in the oxygen-free system there is no indication that the pupa catalyzes any further non-oxidative uptake of carbon monoxide.

The empirical correction factor for the reaction of carbon monoxide with alkali was subtracted from the gas disappearance in the experiment reported in Section 1. The absence of non-oxidative uptake of carbon monoxide by the diapausing pupa enabled an alternative method of correction in subsequent experiments. The thermobarometer was simply flushed with the experimental gas mixture . . . the thermobarometer correction then automatically compensated for the reaction of carbon monoxide with alkali.

In summary, we can state that the rate of gas disappearance in CO-O_2 mixtures is composed of a component due to the reaction of carbon monoxide with alkali, a component due to the combustion of carbon monoxide, and a component attributable to the respiration of the pupa. The experiment reported in the following section was designed to disentangle these latter two phenomena.

3. *Special technique for the concomitant determination of total gas uptake, oxygen uptake and production of carbon dioxide from carbon monoxide*

The total gas uptake (Q_{total}) of a pupa is equal to the oxygen uptake due to respiration ($Q_{O_2}R$), plus the oxygen uptake due to CO-combustion ($Q_{O_2}C$), plus the carbon monoxide uptake due to CO-combustion (Q_{CO}). The latter value is numerically equal to the CO_2 production due to CO combustion ($Q_{CO_2}C$).

$$Q_{total} = Q_{O_2}R + Q_{O_2}C + Q_{CO_2}C$$

From this relationship it is immediately clear that measurements of the total gas uptake and the oxygen uptake due to respiration and combustion combined allow us to calculate the amount of carbon dioxide production due to CO-combustion. In addition, this latter value can be determined directly using carbon^{14}-labeled carbon monoxide.

Each of four diapausing pupae was placed in a modified Warburg vessel and its oxygen uptake in air measured manometrically (Method 2). The pupae were then removed carefully from the Warburg vessels and each was sealed in a modified Fenn volumeter with a gas phase of 82% atm. CO and 18% atm. O_2, as described under Method 3b. In order to introduce a known amount of barium hydroxide, and of $C^{14}O$, and to remove samples for analysis of respiratory gases, the following techniques were employed in elaboration of the operations described in Method 3b.

The index drop of each volumeter was brought to the zero mark by withdrawing gas, using a hypodermic syringe equipped with a #25 blunted needle and lubricated with silicone grease. Then exactly 1.00 ml. of barium hydroxide (0.097 *M* $Ba(OH)_2 \cdot 8\ H_2O$) was added to the sidearm from a second 1-ml. syringe. Using a third 1-ml. syringe, approximately 1 ml. of gas was removed for gas analysis, leaving the meniscus of the index drop at zero. Now 0.800 ml. of the same CO-O_2 mixture was added to each animal vessel and 0.500 ml. to the

thermobarometer. Finally 0.200 ml. of $C^{14}O$ (ca. 36 μc./ml.) was added to each vessel so that the meniscus on each animal-containing volumeter now read 1.000 ml. and that on the thermobarometer read 0.700 ml. The excursions of the index drops were read at intervals of from 1 to 5 hours for a total period of 16 hours. During this same period three 0.200-ml. samples of gas were withdrawn from each volumeter to measure the specific activity of the $C^{14}O$ in the gas phase. As the pupa consumed gas the index drop moved toward zero. Pure oxygen was injected at convenient intervals to return the index drop to 1.000 and to restore the gas phase to its initial composition.

Finally, at the end of the experiment the index drops were all brought to zero by withdrawing gas; a second 1.000 ml. of barium hydroxide was added and the vessels shaken thoroughly to remove the last traces of unabsorbed CO_2. A final 1-ml. sample of gas was taken for analysis of respiratory gases. The vessels were

TABLE I

Comparison in normal diapausing pupae of the rate of oxygen uptake in air with the total gas uptake, oxygen uptake, and $C^{14}O_2$ production in a mixture of CO and O_2*

(a) Animal #	(b) In air Q_{O_2}**	In CO-O_2 mixture*			(f) Per cent of total uptake in CO-O_2 that is attributable to CO uptake
		(c) Q_{total}**	(d) Q_{O_2}**	(e) $Q_{C^{14}O_2}$**	
1085	13	22.7	22.0	1.38	6.1
1086	15	27.1	23.1	1.10	4.1
1087	11	23.7	21.6	1.57	6.6
1088	11	23.1	22.2	0.91	3.9
Average	12	24.1	22.2	1.24	5.2

* The gas mixture was 82% atm. CO and 18% atm. O_2.
** Q values in μl. per gram initial live weight per hour.

removed from the bath and opened. The $BaCO_3$ was plated out on a millipore filter, oven-dried, and transferred to the gas-generating chamber on the manifold; the CO_2 was generated with concentrated H_2SO_4, introduced into the counting chamber and counted.

The rates of uptake of total gas, oxygen, and carbon[14] dioxide are recorded for each of four animals in Table I, together with the corresponding rate of oxygen uptake in air. These data are representative of the results obtained with 48 pupae in 12 experiments essentially similar to the one being described.

4. *The stimulation of respiration by carbon monoxide*

Attention is first directed to columns b, c, and d of Table I. The average Q_{O_2} *in air* was 12 μl./gram/hour (column b). In the presence *of the CO-O_2* mixture the Q_{total} was almost exactly twice this value (column c). However, the Q_{O_2} *in this mixture,* as determined by direct gas analysis (column d), was nearly as high as the Q_{total} in this mixture. Therefore, the stimulation of gas uptake in the presence of CO and O_2 is due almost entirely to an increase in the rate of the true respiration of the pupa. (It should be emphasized that the term "respiration"

refers to the normal metabolic process and does not include the uptake of carbon monoxide and oxygen associated with carbon monoxide-combustion.)

5. *The combustion of carbon monoxide to carbon dioxide*

Columns c and d of Table I show that in the case of each pupa the oxygen uptake in the CO-O_2 mixture is not quite as large as the total gas uptake. On the average the total gas uptake is about 2 μl./gram/hour greater than the average oxygen uptake. This discrepancy, together with the data reported in Section 1, suggests that carbon monoxide is being oxidatively removed from the vessels.

TABLE II

Calculation of total CO_2 that came from CO based on isotopic measurements

(a) Animal #	(b) *cpm per 0.200 ml. sample of original gas mixture**	(c) *cpm per 0.200 ml. of CO (calculated)	(d) *Total cpm in expired CO_2	(e) μl. of CO_2 that came from CO
Thermobarometer	112,000	92,000	−31	−0.068
1085	250,000	205,000	157,195	154
1086	147,000	121,000	54,324	101
1087	174,000	143,000	105,145	154
1088	53,400	43,800	19,466	89

* The values recorded have all been corrected for background activity (*ca* 500 cpm).
** The gas mixture was 82% atm. CO and 18% atm. O_2.

This conclusion is proven beyond reasonable doubt by the results recorded in column e. It is clear that $C^{14}O_2$ is being produced from $C^{14}O$ during the experiment at a rate of about 1.2 ± 10% μl./gram/hour. This amount of gas, together with the oxygen uptake (from gas analysis), accounts completely for the total gas uptake in the CO-O_2 mixture.

These data are presented in further detail in Table II. The background level of radioactivity in the $BaCO_3$ from the thermobarometer (column d) assures us that we are not dealing with a non-specific trapping of $C^{14}O$ in the alkali. Further isotopic experiments (Harvey and Smetana, unpublished observations) demonstrated that neither the catalytic effect of water on CO-combustion nor the solubility of $C^{14}O$ in the tissues of the pupae contributed in any way to the results reported in this paper.

6. *The fate of C^{14}-labeled compounds within the pupa*

a) *Under aerobic conditions*

At the end of the experiment described in Sections 3, 4 and 5, two pupae (nos. 1086 and 1087) were immediately desiccated under high vacuum for half a day. The gases that came from the pupae during this treatment were trapped in $Ba(OH)_2$. The $BaCO_3$ was treated with acid and the generated CO_2 was counted. The $C^{14}O_2$ given off in this way was added to that given off during the experiment and contributes to the $Q_{C^{14}O_2}$ reported in column e of Table I.

The desiccated remains of the pupa were ground up and oven-dried. Two

TABLE III

The fate of carbon^{14}-labeled compounds formed within the pupa during exposure to $C^{14}O$ under aerobic and anaerobic conditions

Animal	Condition during experiment	$C^{14}O_2$ (μl.) given off during experiment	$C^{14}O_2$ (μl.) distilled from pupa after experiment	C^{14} compounds retained by pupa after distillation	
				Non-acidified residue	Acidified residue
1086	aerobic	80	10	11	5.6
1087	aerobic	127	21	5.9	2.0
1094	anaerobic	7.4	0.14	<0.4	<0.4
1095	anaerobic	11.1	0.13	<0.4	<0.4

* Expressed as μl. of $C^{14}O$.

aliquots were taken—one was counted directly and the other was acidified with 6 N HCl, washed with water, re-dried and counted in the same counter. Expressed as μl. of $C^{14}O$, there was an average of 8 μl. trapped in the pupa as calculated from the untreated aliquot, and an average of 4 μl. fixed as calculated from the acidified aliquot (Table III). Thus the reactions of CO and CO_2 combined could have resulted at most in the fixation of 4 μl. of $C^{14}O$.

b) *Under anaerobic conditions*

It is possible that the fixation of $C^{14}O$ is masked by the immediate oxidation of the resulting carbonyl compound by some aerobic process. To favor the accumulation of carbonyl compounds the following procedure was employed.

Each of two cannulated, brainless diapausing pupae was sealed in a volumeter containing $Ba(OH)_2$ with a gas phase of pure CO that had been passed through Fieser's solution (Fieser, 1924) (to assure the complete absence of oxygen from the gas phase), lead acetate, and water. The pupae were allowed to equilibrate for seven hours so that they might use up the last traces of oxygen dissolved within their tissues (Harvey and Williams, 1958a). Then $C^{14}O$ was added to give a final specific activity of 1 million cpm/ml. After 18 further hours each pupa was removed and its gases distilled into $Ba(OH)_2$ as described in the preceding section. The residue of each pupa was dried and divided into an acidified and an un-acidified fraction, each of which was plated out and counted. Each of the four aliquots yielded the normal background count (for this counter) of 18 to 19 cpm. Since this method is sensitive enough to detect the fixation of 0.4 μl. of gas, it is clear that no more than this amount of $C^{14}O$ was fixed under anaerobic conditions (Table III).

c) *The discharge of $C^{14}O_2$ from pupae*

When the experiment described in Sections 3, 4 and 5 was terminated, each of two pupae (nos. 1085 and 1088) was placed in a sealed container with $Ba(OH)_2$. The $BaCO_3$ was collected during each of three intervals from each pupa. The CO_2 was generated with acid and counted. The rate of discharge of $C^{14}O_2$ during and after the experiment is plotted as a function of the time after the ex-

periment in Figure 4. It is clear that the rate of release of $C^{14}O_2$ declines rapidly and after 4 days almost all of the labeled gas has been expired. This $C^{14}O_2$ given off after the experiment was added to that collected during the experiment in the calculations of $Q_{C^{14}O_2}$ recorded for these pupae in Table I.

7. CO-combustion and the level of metabolism

It is possible to vary the level of metabolism in a diapausing pupa merely by rupturing the integument. Such injured pupae consume oxygen at rates several times that of uninjured individuals (Schneiderman and Williams, 1953). This

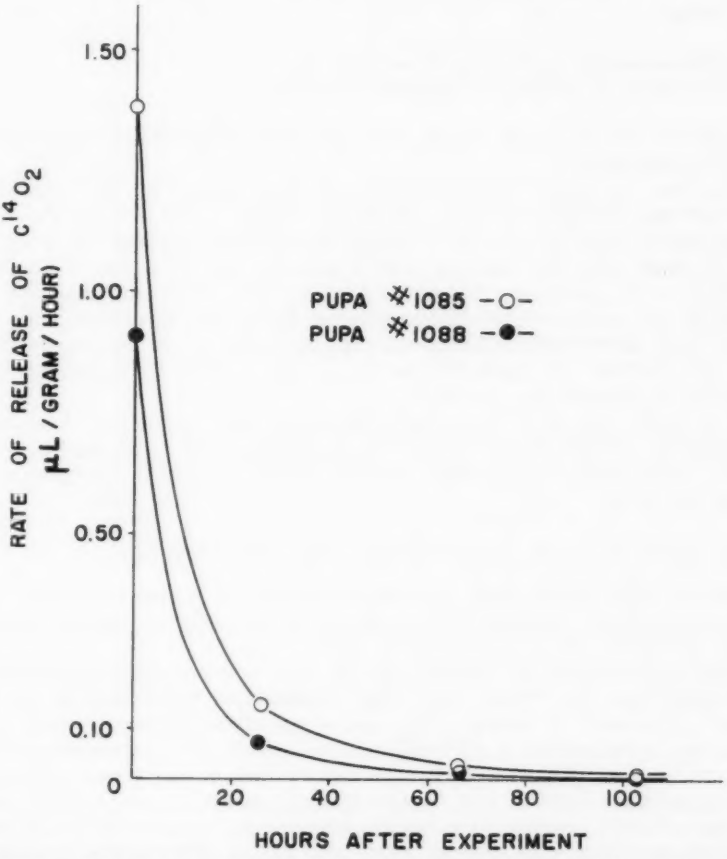


FIGURE 4. The rate of release of $C^{14}O_2$ from diapausing pupae, during exposure to a $C^{14}O$-O_2 mixture (plotted at 0 hours), and during the subsequent period after the exposure was terminated. It is seen that the rate of $C^{14}O_2$ expiration follows a decay function, and that by four days after the termination of exposure all of the radioactive gas has been expired.

TABLE IV

The rate of oxygen uptake of injured pupae in air compared with their total gas uptake and $C^{14}O_2$ production in a mixture of CO and O_2

Animal	In air Q_{O_2}**	In CO-O_2 mixture*		Per cent of total gas uptake that is attributable to CO-combustion
		Q_{total}**	$Q_{C^{14}O_2}$**	
943	76	91	3.22	3.5
944	26	37	1.12	3.0
963	36	38	1.41	3.7
Average	46	55	1.92	3.5

* The gas mixture was 82% atm. CO and 18% atm. O_2.
** Q values in μl. per gram initial live weight per hour.

phenomenon allows one to inquire into the relationship between metabolic rate and CO-combustion.

For this experiment each of three pupae was injured by the establishment of a terminal abdominal window (Williams, 1952). After a week the rate of oxygen uptake was measured in air, using the modified Warburg technique.

The pupae were then exposed to 82% atm. CO and 18% atm. O_2 exactly as described in the experiment in Section 3, except that no gas samples were withdrawn for gas analysis. The $C^{14}O_2$ given off during the experiment and during the 82 hours immediately following the experiment was plated out, generated with acid, and counted. In Table IV the Q_{O_2} in air, and the Q_{total} and the $Q_{C^{14}O_2}$ in the CO-O_2 mixture are presented.

It is clear from these somewhat preliminary data that carbon monoxide is combusted at a slightly higher rate in the injured pupa than in the uninjured pupa. There is some indication that there may be a linear increase in $Q_{C^{14}O_2}$ as a function of Q_{O_2} in air.

8. *The inhibition of the injury metabolism by carbon monoxide*

Having dealt quantitatively with the combustion of carbon monoxide in both normal and injured diapausing pupae, we are in a position to evaluate the effects of this gas on the true respiration of the pupa.

The rate of oxygen consumption of each of 24 brainless diapausing pupae was first measured in air (Method 2). The individuals were then divided into three groups and treated as follows. The rate of gas disappearance of each of nine pupae was re-determined in a CO-O_2 mixture of 90% atm. carbon monoxide and 10% atm. oxygen ($CO/O_2 = 9/1$); the rate for each of nine pupae in 93% atm. carbon monoxide and 7% atm. oxygen ($CO/O_2 = 13/1$); and (making use of the high pressure technique) the rate for each of six pupae, in 500% atm. carbon monoxide and 21% atm. oxygen ($CO/O_2 = 24/1$). The results in each case were corrected for the reaction of carbon monoxide with alkali but not for the combustion of CO to CO_2 (see Discussion (3) for correction for CO-combustion).

Similar determinations were made on a total of ten brainless diapausing pupae

which, one week previously, had been subjected to a large leg injury (Schneiderman and Williams, 1953).

The results are summarized in Figure 5 along with data from the pupae exposed to a mixture of 82% atm. carbon monoxide and 18% atm. oxygen ($CO/O_2 = 5/1$) described in Sections 3 and 5 of the Results. (For simplicity, the results in the CO-O_2 mixture of 13/1 are not presented in the Figure.)

The ratio between the rates of decrease in gas volume in CO-O_2 mixtures and in air have been computed for each animal and plotted as a function of the corre-

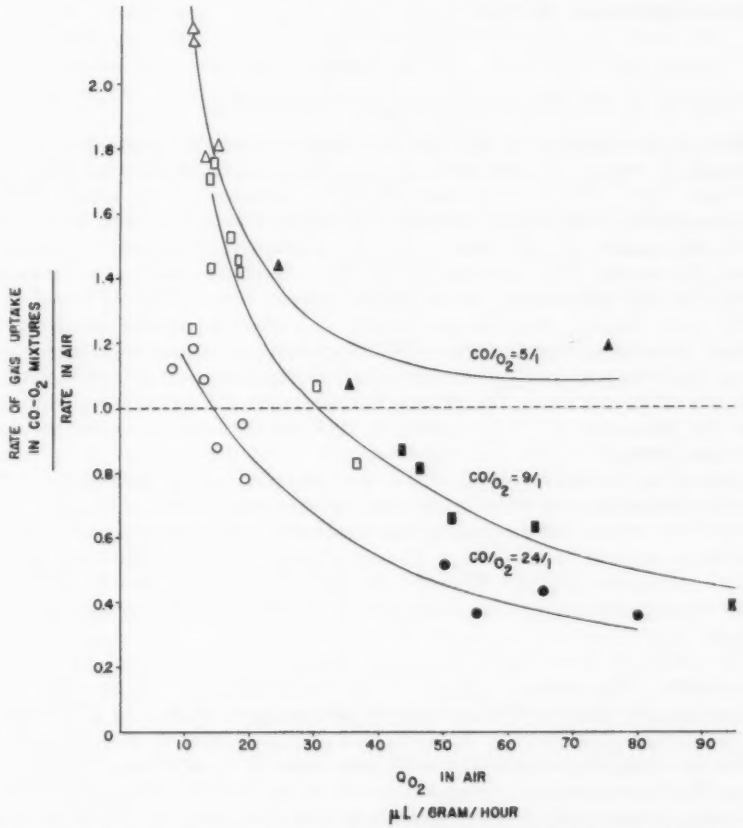


FIGURE 5. For a series of uninjured and injured pupae of the Cecropia silkworm, the relative rates of gas uptake in carbon monoxide *versus* air are plotted as a function of the Q_{O_2} of the corresponding animals in air. Each datum has been corrected for the reaction of carbon monoxide with alkali but not for carbon monoxide-combustion. All points above the dotted line signal an acceleration of gas uptake by carbon monoxide. Points below this line signal a depression by carbon monoxide. The open points are uninjured pupae; the solid points are injured pupae.

sponding Q_{O_2} in air. It will be observed that for animals exposed to the mixture of 82% atm. CO and 18% atm. O_2 this ratio is greater than 1, no matter what the prior Q_{O_2} in air. This means that the gas uptake is invariably higher in the presence of this CO-O_2 mixture than it is in air. When the amount of carbon monoxide in the mixture is increased (lower curves) it will be noted that this ratio remains greater than 1 only for pupae exhibiting a low Q_{O_2} in air. In pupae whose metabolism has been enhanced by injury, the ratio is invariably less than 1, indicating that the rate of gas uptake is inhibited. Finally in each of the lower curves there is an intermediate level of metabolism where carbon monoxide neither stimulates nor inhibits.

Discussion

1. *Carbon monoxide-combustion and cytochrome oxidase*

Most of the evidence in the literature suggests that the combustion of carbon monoxide by oxygen is mediated by cytochrome oxidase (Breckenridge, 1953; Black and Tyler, 1959b). The most damaging evidence for this hypothesis is the augmentation of CO-combustion in light (Stannard, 1940; Black and Tyler, 1959a) where, presumably, the cytochrome oxidase-carbon monoxide complex is very unstable. However, Black and Tyler (1959a) attempt with some success to account for this light-stimulation of CO-oxidation. Stannard (1940) and Chance (1949) have implied that the combustion of carbon monoxide is mediated by catalase. However, Breckenridge (1953) exposed several purified enzyme preparations, including catalase, cytochrome *c* and diaphorase, to mixtures of 80% atm. $C^{14}O$ and 20% atm. O_2. He showed that only cytochrome oxidase was able to cause the formation of $C^{14}O_2$; moreover, this activity was augmented as the enzyme was purified.

As a third alternative, Wald (1959) has proposed that an intermediate (RH) might be carbonylated to the corresponding aldehyde (RCOH). The latter might subsequently reduce water, yielding two hydrogens, while the aldehyde was being oxidized to the acid (RCOOH). The hydrogens would be available as substrate for the cytochrome system while the acid could be decarboxylated to yield carbon dioxide and thereby regenerate the intermediate (RH) which would thus act as a catalyst.

The results of the present study argue against the formation of a carbonylated intermediate. The failure to detect carbon^{14}-labeled material in the dried residue of the anaerobic pupa (Table III) argues that no direct fixation of $C^{14}O$ occurred. The remote possibility exists, however, that the small amount of $C^{14}O_2$ given off during the anaerobic experiment may have been the oxidation product of some carbonylated intermediate. Only a trace of carbon^{14}-labeled compound other than $C^{14}O_2$ was found in the aerobic experiment where as many as 127 μl. of this gas were formed (Table III). This small aerobic carbon^{14} fixation was undoubtedly due to the fixation of some endogenous $C^{14}O_2$ produced within the pupa. Finally, it will be recalled that there was no manometric evidence for carbon monoxide uptake by the anaerobic pupa (Fig. 3).

These findings fail to support Wald's theory of the carbonylated intermediate. Neither do they entirely disprove it, since the fixation of less than 0.4 μl. (9 μ

moles) of carbon monoxide could not be detected by this procedure. Possibly an accumulation of less than 9 μ moles of intermediate would be sufficient for Wald's carbonylation-catalytic process.

The simplest interpretation of these data is that both $C^{14}O$ and O_2 react directly with cytochrome oxidase to form $C^{14}O_2$. Some of the $C^{14}O_2$ is exhaled immediately as a gas, some goes into solution in the tissues, some exchanges with tissue bicarbonate, eventually to be given off as a gas, while presumably some of the $C^{14}O_2$ is fixed (Table III).

This interpretation would seem to require either that CO and O_2 react in sequence with cytochrome oxidase or, more simply, that they react simultaneously with this enzyme. This second prospect implies that there is more than one combining site on the cytochrome oxidase molecule. Wald and Allen (1957) find that, in the reaction of carbon monoxide with cytochrome oxidase, the coefficient n is greater than 1 in the Hill equation. This finding implies an interaction at the catalytic site between more than one molecule of carbon monoxide on cytochrome oxidase which, in turn, implies the presence of more than 1 heme per active molecule.

Ambe and Venkataraman (1959) report that in highly purified, homogeneous preparations of cytochrome oxidase there is but one heme per monomer. However, they also find one copper atom per heme group. Of the greatest importance was their finding that their monomer displayed no cytochrome oxidase activity unless phospholipide was added.

These seemingly contradictory observations are reconciled by the hypothesis that several individual units (monomers) of cytochrome oxidase are oriented on the phospholipide in such a way that interaction between two or more monomers is possible.

Although there are many other interpretations of these data, the combustion of carbon monoxide by molecular oxygen is a fact which must be accounted for, and an instrument which must not be overlooked, in efforts to understand the mechanism by which cytochrome oxidase activates oxygen.

2. *Carbon monoxide and the metabolism of diapausing pupae*

The diapausing pupa is particularly responsive to carbon monoxide. In no other animal known to us has CO-stimulation of respiration been clearly demonstrated.

CO-combustion, on the other hand, is well-known in a variety of animals. In Cecropia pupae this reaction accounts for but from 5 to 7% of the total gas uptake in CO-O_2 mixtures, in marked contrast to the 57% found in frog muscle (Clark, Stannard and Fenn, 1950). In developing sea urchin eggs the values range from 10 to 43% (Black and Tyler, 1959a). It is tempting to speculate that all aerobic cells are capable of catalyzing CO-oxidation and that the magnitude of the phenomenon varies from animal to animal depending perhaps, as Breckenridge (1953) has suggested, on the state of oxidation of their cytochrome oxidase.

3. *Carbon monoxide-inhibition of the true respiration of injured pupae*

When the gas uptake due to CO-combustion is subtracted from the total gas uptake, the true respiration in CO-O_2 mixtures is revealed. From Tables I and

IV we see that (with a $CO\text{-}O_2$ ratio of 5 to 1) we must subtract 1.8 μl./gram/hour for CO-combustion of normal diapausing pupae and 2.9 μl./gram/hour for injured pupae. Extensive semi-quantitative experiments (Harvey, unpublished) show that at higher $CO\text{-}O_2$ ratios the gas uptake due to CO-combustion is somewhat less, though still easily detectable with radioactive material. Since the correction is small compared to the total gas uptake of injured pupae, the values mentioned above are sufficiently accurate for our present purposes.

Thus the effects of $CO\text{-}O_2$ mixtures on the true respiration of pupae follow the same functions as those presented in Figure 5 except that each curve is shifted for from 0.2 to 0.4 units toward the abscissa. This means that the stimulation of true respiration by carbon monoxide is somewhat less than that indicated by considerations of the total gas uptake, and the inhibition of true respiration is somewhat greater.

This CO-inhibition of the true respiration of injured pupae is of considerable theoretical interest. Harvey (1956) reports that the total gas uptake of injured pupae is inhibited by carbon monoxide—a finding subsequently confirmed by Kurland and Schneiderman (1959). We can now state that the true respiration of injured pupae is inhibited to an even greater extent. Harvey and Shappirio (unpublished observations) have shown that this augmented CO-sensitivity of the injury-enhanced true respiration of pupae is attributable to an increase in the activity of certain cytochrome enzymes.

I would like to express my appreciation to Professor Carroll M. Williams for his critical reading of the manuscript, and to Mr. Frank Smetana and Mr. John Steen for their technical advice and assistance.

Summary

1. After due correction for complicating reactions, the rate of total gas uptake of normal diapausing pupae of the Cecropia silkworm in a carbon monoxide-oxygen mixture of 5 to 1 is found to be stimulated approximately 2-fold over the rate in air.

2. Ninety per cent of this *extra* gas uptake is due to a stimulation of the true respiration of the pupa.

3. The remaining ten per cent is due to the combustion of carbon monoxide to carbon dioxide.

4. The combustion of carbon monoxide is increased slightly when the metabolism of the pupa is enhanced following integumentary injury.

5. There is a slight fixation of carbon-14 in pupae under aerobic conditions, presumably as a result of fixation of $C^{14}O_2$ manufactured from $C^{14}O$ by the pupa.

6. There is no detectable fixation of $C^{14}O$ in pupae exposed to this gas under anaerobic conditions.

7. After correction for carbon monoxide-combustion, it is clear that the enhanced oxygen uptake following integumentary injury of diapausing pupae is inhibited by carbon monoxide.

8. This carbon monoxide-inhibition is directly proportional to the enhancement of oxygen uptake following injury.

LITERATURE CITED

AMBE, K. S., AND A. VENKATARAMAN, 1959. Depolymerization of cytochrome oxidase to a water-soluble monomeric protein. *Biochem. Biophys. Res. Comm.*, **1**: 133–137.

BARTON, J. A., AND D. PARRISH, 1956. Tritium-gas dispenser. *Nucleonics*, **14**, No. 6: 85–86.

BERNSTEIN, W., AND R. BALLENTINE, 1950. Gas phase counting of low-energy β-emitters. *Rev. Sci. Instr.*, **21**: 158–162.

BLACK, R. E., AND A. TYLER, 1959a. Effects of fertilization and development on the oxidation of carbon monoxide by eggs of *Strongylocentrotus* and *Urechis* as determined by use of C^{18}. *Biol. Bull.*, **117**: 443–453.

BLACK, R. E., AND A. TYLER, 1959b. Cytochrome oxidase and CO-oxidation in eggs of the sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull.*, **117**: 454–457.

BRECKENRIDGE, B., 1953. Carbon monoxide oxidation by cytochrome oxidase in muscle. *Amer. J. Physiol.*, **173**: 61–69.

BUCK, J., AND M. KEISTER, 1955. Cyclic CO_2 release in diapausing *Agapema* pupae. *Biol. Bull.*, **109**: 144–163.

CHANCE, B., 1949. The primary and secondary compounds of catalase and methyl or ethyl hydrogen peroxide. *J. Biol. Chem.*, **179**: 1341–1369.

CLARK, R. T., JR., J. N. STANNARD AND W. O. FENN, 1950. The burning of CO to CO_2 by isolated tissues as shown by the use of radioactive carbon. *Amer. J. Physiol.*, **161**: 40–46.

DALY, J. M., 1954. Stimulation of respiration by carbon monoxide. *Arch. Biochem. Biophys.*, **51**: 24–29.

FENN, W. O., 1935. The differential volumeter for the measurement of cell respiration and other processes. American Instrument Co., Inc., publication.

FIESER, L., 1924. A new absorbent for oxygen in gas analysis. *J. Amer. Chem. Soc.*, **46**: 2639–2647.

HARVEY, W. R., 1956. The effect of carbon monoxide and diphtheria toxin on the injury metabolism of diapausing Cecropia silkworms. *Anat. Rec.*, **125**: 556.

HARVEY, W. R., AND C. M. WILLIAMS, 1958a. Physiology of insect diapause. XI. Cyanide-sensitivity of the heartbeat of the Cecropia silkworm with special reference to the anaerobic capacity of the heart. *Biol. Bull.*, **114**: 23–35.

HARVEY, W. R., AND C. M. WILLIAMS, 1958b. Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm. *Biol. Bull.*, **114**: 36–53.

KURLAND, C. G., AND H. A. SCHNEIDERMAN, 1959. The respiratory enzymes of diapausing silkworm pupae: A new interpretation of carbon monoxide-insensitive respiration. *Biol. Bull.*, **116**: 136–161.

LILIENTHAL, J. L., JR., 1950. Carbon monoxide. *Pharmacol. Rev.*, **2**: 324–354.

SCHOLANDER, P. F., 1947. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. Biol. Chem.*, **167**: 1–17.

SCHNEIDERMAN, H. A., AND N. FEDER, 1954. A respirometer for metabolic studies at high gaseous pressures. *Biol. Bull.*, **106**: 230–237.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1953. The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **105**: 320–334.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1955. An experimental analysis of the discontinuous respiration of the Cecropia silkworm. *Biol. Bull.*, **109**: 123–143.

SHAPPIRIO, D. G., AND C. M. WILLIAMS, 1957. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B*, **147**: 218–232.

STANNARD, J. N., 1940. An analysis of the effect of carbon monoxide on the respiration of frog skeletal muscle. *Amer. J. Physiol.*, **129**: 195–213.

THIMANN, K. V., C. S. YOCUM AND D. P. HACKETT, 1954. Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. *Arch. Biochem. Biophys.*, **53**: 239–257.

WALD, G., 1959. Evolution of respiratory mechanisms. Abstract, meeting of Amer. Chem. Soc., Div. of Biol. Chem., (April 5–10), Boston, Mass.

WALD, G., AND D. W. ALLEN, 1957. The equilibrium between cytochrome oxidase and carbon monoxide. *J. Gen. Physiol.*, **40**: 593–608.

WARBURG, O., 1927. Über die Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. *Biochem. Zeitschr.*, **189**: 354–380.

WILLIAMS, C. M., 1946. Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

WILLIAMS, C. M., 1952. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.*, **103**: 120–138.

WILLIAMS, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.*, **110**: 201–218.

EXPERIMENTS ON THE NORMAL MEIOTIC BLOCK IN THE OVUM OF TRITURUS VIRIDESCENS[1]

A. A. HUMPHRIES, JR.

Department of Biology, Emory University, Atlanta 22, Georgia

At the time of penetration of the amphibian egg by the sperm, the second division of meiosis is arrested at metaphase. Entrance of the sperm triggers the activation of the blocked division and the second polar body is produced. Much interest has, through the years, been centered about the problem of just what triggers the completion of the division as a part of the "activation" of the egg. Equally fascinating, but largely ignored, is the question of the nature and origin of the meiotic block which is removed by the entrance of the sperm.

The variation in the stage of meiosis customarily reached by the eggs of the major animal groups before fertilization has been known for many years (for references see Wilson, 1925; Just, 1939; Dalcq, 1957; Rothschild, 1956). In the vertebrates, the egg is usually fertilized at metaphase of the second maturation division, and it is commonly stated that the division in unfertilized eggs is blocked at this stage. This statement holds for most amphibian eggs, although variations have been noted for *Siredon* (*Ambystoma*) (Schultze, 1887) and for *Triturus viridescens* (Humphries, 1955, 1956). As will be shown in this paper, the exceptions in *T. viridescens* seem to occur only under certain circumstances.

In earlier work (Humphries, 1956) it was shown that in *Triturus viridescens* the first maturation spindle forms at about the time of ovulation, and the first polar body is extruded soon after the egg enters the oviduct. The second division figure then forms and remains at metaphase until fertilization occurs. Coelomic eggs which, for any reason, are delayed in entering the oviduct, extrude the first polar body in the coelom and show the formation of the second metaphase figure in the same way as do oviducal eggs. However, two of the coelomic eggs in that study had passed the usual stage of arrest and were in anaphase II. It seemed possible that the difference between the oviducal and coelomic environments might be responsible for the escape from the block in these eggs and, further, that the oviducal environment might be a factor in the normal blocking of meiosis in unfertilized eggs. The present report deals with an investigation of these possibilities, using two approaches: (1) ligation of the oviducts at several levels, to retain the eggs abnormally long in the coelom or in certain regions of the oviducts, and (2) maintenance of eggs *in vitro*, with and without jelly coats.

Materials and Methods

Ligation experiments: Adult *Triturus viridescens* females were obtained from ponds near Charlottesville, Virginia, and Franklin and Canton, North Carolina,

[1] Supported by research grant RG-6291 from the United States Public Health Service, an Institutional Grant from the American Cancer Society, and the McCandless Fund of Emory University.

and kept in a refrigerator at about 12° C. until used. The oviducts in this species show a gross differentiation into distinct regions (Adams, 1940) which have been identified, from anterior to posterior, as regions A through E (Humphries and Hughes, 1959). An ostial portion, which is apparently non-secretory, precedes region A. The deposition of jelly about the egg begins as the egg passes through region A. The oviducts were ligated as close to the ostium as possible, or at the posterior limits of the A, C, or D regions. Following ligation, the females were allowed to recover for a period of a few days to two weeks, after which they received a subcutaneous implantation of one or two intact anterior lobes of the pituitary of *Rana pipiens*. If two lobes were used, the second implantation followed the first by 48 hours. Animals were sacrificed and opened 48 or 72 hours after the final pituitary implantation and any eggs which had accumulated in the coelom or oviducts were removed, placed in Bouin's fixative, embedded in Tissuemat, sectioned at 10 microns, and stained with Harris' haemalum and fast green. In the first experiments done, using only animals with oviducts ligated at the ostium, four advances past metaphase II were obtained from a total of 66 eggs which had reached at least metaphase II. Two were from "48-hour animals," and two were from "72-hour animals." It apeared, therefore, that 48 hours was an adequate time interval in which to expect advances and this was used for all remaining ligation experiments. The eggs used in these experiments were obtained from a total of 43 animals. Statistical analysis was made through the use of the contingency chi-square (Mather, 1943), using the Yates correction.

In vitro experiments: Animals used in this part of the study were obtained from lakes near Franklin and Highlands, North Carolina. In this group ovulation was stimulated by the injection into the coelom of 0.25 to 0.40 cc. of chorionic gonadotropin (Antuitrin "S," Parke, Davis, 500 units per cc.). When necessary, a second injection was given, usually 48 hours after the first. Eggs present in the coelom, ostium, and secreting parts of the oviducts were removed following decapitation of the animals, and were placed in solutions in covered syracuse dishes, the bottoms of which, in most instances, had been covered with paraffin to prevent adhesion of eggs to the glass. In some cases, the bottom was covered with filter paper wet with one of the solutions used. About a third of the oviducal eggs were freed mechanically from their jelly, using watchmaker's forceps, before being placed in solution. The solutions employed were Holtfreter's solution, Ringer's solution, and Niu-Twitty solution. Some eggs were kept in small droplets of solution, barely sufficient to cover them, while others were kept in well filled dishes. In the case of eggs kept in droplets, and in the case of those kept on wet filter paper, the covers of the dishes were sealed with Vaseline. Most of the eggs were kept at 20° C. in a water bath for 24 hours, though some were kept for 12 or 48 hours. Fixation and processing were the same as outlined for the previous experiments, except for a few eggs which were stained in toluidine blue for cytochemical study. The eggs used in this part of the study were taken from a total of 49 animals.

The statistical method used was the same as for the results of the ligation experiments.

Observations

Ligation experiments: The stages of meiosis in eggs of the various categories are given in Table I. Ovulation occurs over an extended period in this species;

thus, some of the eggs were probably out of the ovary and in the coelom or oviducts for two days or more prior to fixation, while others may have been released from the ovary only a few minutes before the animal was sacrificed. There is no way of knowing, other than in a general way, based on position in the oviduct and stage of meiosis, just how long a given egg has been out of the ovary. Under the circumstances, one would expect to find the entire spectrum of stages of meiosis, ranging from metaphase I through metaphase II and perhaps beyond. Such a situation is reflected in the distribution shown in Table I.

TABLE I

Stages of meiosis in oocytes removed from coelom and oviducts of operated animals 48 or 72 hours following final pituitary implantation*

Source	Earlier than Metaphase II	Metaphase II	Anaphase II to Telophase II	Meiosis complete
Coelom—ovid. not ligated at ostium	43	81	0	0
Coelom—ovid. ligated at ostium	32	44	0	1
Ostium—ovid. ligated in ostial region	17	35	1	3
Region A—ovid. ligated post. end of "A"	7	14	0	0
Region A—ovid. ligated post. end of "C"	1	10	0	0
Region A—ovid. ligated post. end of "D"	1	0	0	0
Region B—ovid. ligated post. end of "C"	1	0	0	0
Region C—ovid. ligated post. end of "C"	3	167 (51 had no jelly)	0	2**
Region D—ovid. ligated post. end of "C"	1	3	0	0
Region D—ovid. ligated post. end of "D"	2	38	0	0
Coelom—escaped from oviduct—jelly-covered	1	58	0	0
Totals	109	450	1	6

* See text.
** Not jelly-covered.

Meiosis was judged to be complete under one of the following conditions: presence of two polar bodies; presence of two polar bodies plus a single set of single chromosomes (monads) within the egg (Fig. 3); presence of a set of monads within the egg, in the absence of one or both polar bodies. In these eggs the single chromosomes within the egg were never in other than the condensed condition of mitotic chromosomes, nor were they surrounded by a nuclear membrane. In the one case of anaphase II which was observed, single chromosomes were about half-way to the poles and the first polar body was present (Figs. 1 and 2).

Meiosis was found to be advanced or complete only in eggs with no jelly coats. Advances occurred in coelomic eggs, in eggs from the ostial (non-jelly-secreting)

region of the oviducts, and in non-jelly-covered eggs from region C of the oviducts. Region C is a secreting region, and eggs in this region ordinarily have jelly coats deposited by the C region and by more anterior parts of the oviduct, but in a few instances the ligated oviducts became so distended with eggs that eggs which were

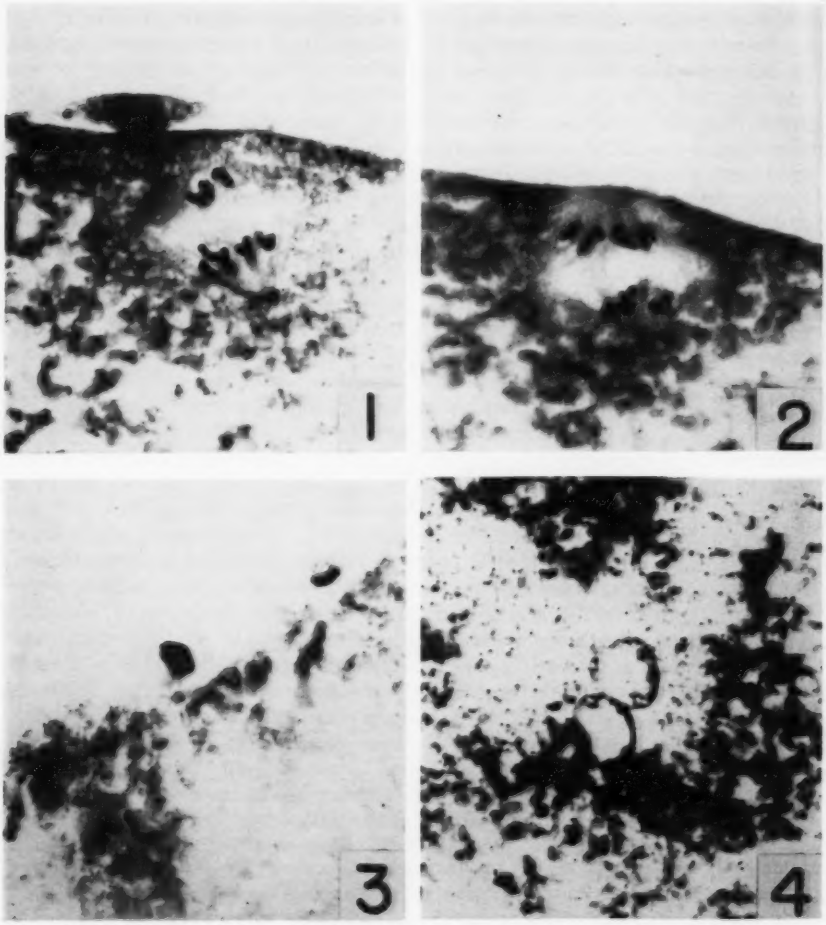


FIGURE 1. Anaphase II with adjacent polar body I, from the ostium of an oviduct ligated as close as possible to the anterior end. × 790.

FIGURE 2. Section adjacent to that shown in Figure 1. × 790.

FIGURE 3. Completed meiosis in an egg from the coelom of an animal with oviducts ligated at the ostia. Three chromosomes of the haploid set of eleven can be seen at the lower left. Portions of polar bodies I and II are visible outside the egg. × 970.

FIGURE 4. Two interphase nuclei in an egg from the coelom kept for 40 hours in Niu-Twitty solution. × 770.

TABLE II

Comparison of number of advances past Metaphase II in eggs with no jelly coats and eggs having jelly coats in ligation experiments

	Metaphase II	Later than Metaphase II	Total
With jelly	229	0	229
Without jelly	130	7	137
Totals	359	7	366

centrally located within the mass apparently never came in contact with the secretory epithelium and obtained no jelly. The eggs from region C in which advances were observed were of this type.

The frequency of advance past metaphase II in jelly-covered and non-jelly-covered eggs is given in Table II. Eggs in stages earlier than metaphase II and eggs which were probably not actually delayed by a ligature (*e.g.*, coelomic eggs taken from an animal whose oviducts were ligated toward the middle or posterior part) are not included. Statistical analysis of the distribution gives $P = < .01$.

In vitro experiments: Information as to the stages found in the 254 eggs of the four types kept *in vitro* is given in Table III. No advances past metaphase II occurred in jelly-covered eggs, and only one doubtful advance occurred in the eggs from which the jelly had been removed. Advances were found in 22% of the eggs never exposed to jelly. Comparison of the frequency of advance in eggs exposed to jelly (including those from which the jelly was removed) with those never exposed to it gives $P = < .001$.

TABLE III

Stages in eggs maintained in vitro (24 hours except in cases noted)

Type of egg	First meiotic division	Metaphase II	Anaphase II or Telophase II	Meiosis complete, condensed chromosomes or none found	Pronucleus or pronuclei	Post-meiotic "mitosis"	Total
Coelomic	0	73 (three for 12 hrs. only)	6 (one for 12 hrs. only)	1	2 (one for 12 hrs., one for 40 hrs.)	11	93
Ostial	0	8	2	1	0	0	11
Oviducal, with jelly	0	103 (four for 12 hrs., eleven for 48 hrs.)	0	0	0	0	103
Oviducal, jelly removed	0	46 (one for 12 hrs., eight for 48 hrs.)	1?	0	0	0	47
Totals		230	9	2	2	11	254

The solution in which the eggs were kept seemed to have no effect on the frequency of advance nor on the "normality" of the divisions, although eggs kept in Ringer's solution could usually be distinguished microscopically from those kept in Holtfreter's or Niu-Twitty solution by their "more dense" appearance. Also, the amount of fluid surrounding the egg seemed to be of little, if any, importance.

In eleven of the twenty coelomic eggs in which advance occurred, post-meiotic mitotic activity was occurring. Such "mitoses" often occurred well beneath the egg surface, as in a cleavage division, in contrast to meiotic divisions, which usually occur at the surface. In several instances it appeared that a haploid set of monads was being randomly distributed to the poles, since, even at anaphase, the total number of chromosomes on the spindle was only the haploid number. There were no asters nor was there any evidence of attempted cytoplasmic division. Such "mitosis" probably accounts for the "pronuclei" which were seen in one egg (Fig. 4).

Discussion

There are two general alternatives which may be suggested as possible explanations of the source of meiotic arrest in the amphibian egg. One is that the source is within the egg itself, the other is that the block is somehow imposed upon the egg through some aspect of the environment. The former seems to be the explanation in eggs of *Habrobracon*, where von Borstel (1957) has found evidence that blockage is under genetic control. A third possibility is that a combination of internal and external factors is involved. Not nearly enough is known of either the physiology of the amphibian oocyte or of its environment to allow a ready choice between the alternatives.

Probably the explanation most attractive at present is that proposing a blocking mechanism intrinsic to the egg. The results of the present study should be examined from this point of view. If this is done, certainly one feature of the data seems to agree strongly with the hypothesis of an intrinsic blocking mechanism, and that is the fact that the great majority of eggs which had progressed as far as the second division had not proceeded beyond metaphase of that division. This was true even of non-jelly-covered eggs, the only type in which the block was evaded. The implication is that the process normally stops at this stage, and that, rather infrequently, it is capable of proceeding to completion. If nothing else, it appears that there is a lengthy pause at metaphase. However, in many cases there was little indication as to how long the divisions had been in progress, and undoubtedly some of the eggs had only recently reached metaphase II, and could not have been expected to have advanced beyond that stage. If intrinsic blockage is assumed, however, then what mechanism could account for the evasion of the block in the eggs of this study? It is possible that degenerative changes within the eggs somehow triggered the completion of the second division, but if this is the case, the jelly deposited by the oviducts must protect against such changes or the effects of such changes, since no jelly-covered egg showed advance. Also, eggs which are laid, but not fertilized, will degenerate in their jelly coats without advancing past the normal stage of blockage.

Under the assumption of intrinsic blockage, perhaps the most acceptable hypothesis for explaining the present results is that some aspect of the environment, either

the natural coelomic or ostial environment or the artificial ones, was effective in stimulating completion of the division, perhaps through stimulation of the division apparatus directly or through a general stimulation of some aspect of the metabolism of the egg. Again, however, the jelly coats seem to protect, this time against a possible external stimulatory effect, since jelly-covered eggs which escaped from the oviducts back into the coelom, or which were kept *in vitro* for as long as 48 hours, were never more advanced than metaphase II.

The present results should also be viewed in the light of the possibility that normal meiotic blockage is a function of some factor of the environment of the egg, and not of the egg itself. Under this hypothesis, meiosis might be considered to be a process which, once having been initiated, would, in the absence of outside interference, continue to completion. If an outside inhibitor is considered, the oviducal secretions, specifically the jelly layers deposited about the oocyte, seem to be especially suspect as a source of inhibition. Previous workers (Bataillon and Tchou-Su, 1930; Dalcq *et al.*, 1936) have considered the oocytes of anuran amphibians to be "intoxicated" with carbon dioxide, presumably related to the secretions of the oviducts. Such an "intoxication" might itself be the cause of meiotic blockage, which would perhaps be thought of as an indirect effect of the oviducal jelly.

There is also the possibility of a more direct effect of the oviducal secretions. The first division is usually completed and the second division spindle is usually formed in the anterior part of the oviduct (Humphries, 1956). The division spindle is located with one pole at the egg surface, where it might be unusually sensitive to an outside effect. These facts make the idea of a possible effect of oviducal secretion worth consideration, especially in the light of the results of the present study. The lack of advance in jelly-covered eggs is itself important in this consideration, but the fact that only one doubtful case of escape from blockage occurred in the eggs from which the jelly was removed before their stay *in vitro* suggests that mere exposure to the jelly, not necessarily its presence, may somehow be effective in preventing advance. Recent studies on the histochemistry of the oviducts of urodeles (Kambara, 1956a, 1956b, 1957a, 1957b; Humphries and Hughes, 1959) have shown the presence of acid mucopolysaccharide in the anterior regions of the oviducts, while Minganti (1955) has reported a heparin-like anticoagulant activity of amphibian egg jelly. Heilbrunn and his co-workers, as well as others, have repeatedly shown that compounds of this type are capable of antimitotic activity and that they are of widespread occurrence (see especially Heilbrunn, 1956, and Heilbrunn *et al.*, 1957). Others, working with eggs of the sea urchin, have apparently implicated mucopolysaccharides in general inhibitory activity prior to fertilization (Runnström and Immers, 1956). Certainly the present findings require a serious consideration of the possibility of a direct or indirect effect of the oviducal secretions.

The exploration of the problem of meiotic blockage thus far does not allow a choice between the major alternative explanations. It is apparent that the jelly layers are of significance with regard to blockage under the conditions of these experiments, and they may be involved, as well, in blockage as it occurs under natural conditions. However, the jelly may be important only as an insulator from the environment. Were the jelly the source of the meiotic block, one would expect

all eggs unexposed to jelly to complete meiosis if given ample time and suitable environment. If we assume that these conditions were met by the *in vitro* experiments reported here, in which most such eggs showed arrest, then it is not possible to maintain that the jelly is the sole factor in meiotic arrest, although it may play an important ancillary role, such as the maintenance of an already established block.

Summary

1. The oviducts of the newt, *Triturus viridescens*, were ligated at several levels to retain oocytes from 48 to 72 hours in the coelom or in certain regions of the oviducts. Eggs were collected and studied to determine the stage of meiosis, with the finding that advances past metaphase II, the normal stage of arrest, occurred at a low frequency, but were confined to eggs never exposed to the jelly secreted by the oviducts.

2. Twenty-two per cent of the eggs removed from the coelom or ostium of the oviduct and kept *in vitro* advanced past metaphase II, but eggs with jelly coverings did not advance. One doubtful advance was observed in an egg from which the jelly had been removed prior to its stay *in vitro*. Some "advanced" eggs exhibited post-meiotic "mitotic" activity.

3. The general problem of meiotic blockage in the egg is discussed. The jelly secreted by the oviducts is of significance with regard to meiotic arrest under the conditions of the experiments reported, and may play some role in normal arrest. The evidence does not, however, allow a decision as to the source of meiotic inhibition.

Literature Cited

Adams, A. Elizabeth, 1940. Sexual conditions in *Triturus viridescens*. III. The reproductive cycle of the adult aquatic form of both sexes. *Amer. J. Anat.*, **66**: 235–275.

Bataillon, E., and Tchou-Su, 1930. Etudes analytiques et expérimentales sur les rythmes cinétiques dans l'oeuf (*Hyla arborea, Paracentrotus lividus, Bombyx mori*). *Arch. de Biol.*, **40**: 439–540.

Dalcq, Albert, 1957. Introduction to General Embryology. Oxford University Press, London.

Dalcq, Albert, Jean Pasteels and Jean Brachet, 1936. Données nouvelles (*Asterias glacialis, Phascolion strombi, Rana fusca*) et considerations theoriques sur l'inertie de l'oeuf vierge. *Mém. du Musée Royal d'Histoire Naturelle de Belgique*. Deuxième série, fasc. 3, pp. 881–912.

Heilbrunn, L. V., 1956. The Dynamics of Living Protoplasm. Academic Press, New York.

Heilbrunn, L. V., W. L. Wilson, T. R. Tosteson, E. Davidson and R. J. Rutman, 1957. The antimitotic and carcinostatic action of ovarian extracts. *Biol. Bull.*, **113**: 129–134.

Humphries, A. A., Jr., 1955. Experimental removal of metaphase II inhibition in ova of the newt, *Triturus viridescens*. ASB Bull., **2**: 7.

Humphries, A. A., Jr., 1956. A study of meiosis in coelomic and oviducal oocytes of *Triturus viridescens*, with particular emphasis on the origin of spontaneous polyploidy and the effects of heat shock on the first meiotic division. *J. Morph.*, **99**: 97–136.

Humphries, A. A., Jr., and W. N. Hughes, 1959. A study of the polysaccharide histochemistry of the oviduct of the newt, *Triturus viridescens*. *Biol. Bull.*, **116**: 446–451.

Just, E. E., 1939. The Biology of the Cell Surface. Blakiston, Philadelphia.

Kambara, Shunichi, 1956a. Histochemical studies on the distribution of phosphatases in the oviduct of the newt, *Triturus pyrrhogaster*. *Annot. Zool. Japon.*, **29**: 86–90.

Kambara, Shunichi, 1956b. Histochemical studies on the distribution of mucopolysaccharides in the oviduct of the newt, *Triturus pyrrhogaster*. *Annot. Zool. Japon.*, **29**: 146–150.

Kambara, Shunichi, 1957a. Histochemical studies on the distribution of phosphatases and mucopolysaccharides in the urogenital organs of the newt, *Triturus pyrrhogaster*. *Annot. Zool. Japon.*, **30**: 143–149.

KAMBARA, SHUNICHI, 1957b. Metachromatically staining material in epithelial cells of the oviduct of the newt, *Triturus pyrrhogaster*. *J. Fac. Sci., Univ. Tokyo. Sec. IV*, **8**: 145–148.

MATHER, K., 1943. Statistical Analysis in Biology. Interscience Publishers, New York.

MINGANTI, A., 1955. Chemical investigations on amphibian egg jellies. *Exp. Cell Research*, Suppl., **3**: 248–251.

ROTHSCHILD, LORD, 1956. Fertilization. Methuen & Co., Ltd., London.

RUNNSTRÖM, J., AND J. IMMERS, 1956. The role of mucopolysaccharides in the fertilization of the sea urchin egg. *Exp. Cell Research*, **10**: 354–363.

SCHULTZE, O., 1887. Untersuchungen über die Reifung und Befruchtung des Amphibieneies. *Zeitschr. wiss. Zool.*, **45**: 177–226.

VON BORSTEL, R. C., 1957. Nucleocytoplasmic relations in early insect development. *In:* The Beginnings of Embryonic Development (Albert Tyler, R. C. von Borstel and Charles B. Metz, eds.) pp. 175–199. American Association for the Advancement of Science, Washington, D. C.

WILSON, EDMUND B., 1925. The Cell in Development and Heredity. Macmillan, New York.

HUMIDITY REACTIONS OF SOME AQUATIC ISOPODS IN THE AIR

KARI LAGERSPETZ AND AILA LEHTONEN

Department of Zoology and Lohm Marine Biological Station, University of Turku, Turku, Finland

In nearly all groups of terrestrial invertebrates the occurrence of behavioral reactions to the humidity of air has been established. In addition to the different groups of terrestrial arthropods mentioned, *e.g.*, by Lagerspetz and Jäynäs (1959), earthworms (Heimburger, 1924) seem to exhibit humidity reactions. In an amphibious vertebrate, *Triton*, behavioral reactions to the humidity of the air have also been found to occur (Czeloth, 1930).

For aquatic invertebrates, the present authors have been unable to find in the literature any data on their possible humidity reactions when transferred to air. Maxwell (1897) found that specimens of *Nereis*, when placed on the border of moist and dry sand, more often crept to the moist side. In this, as well as in some other cases, the animals apparently reacted to the contact stimuli, probably by contact chemoreception. In any case, the results of such experiments do not exclude the possibility of contact reception. The field experiments on the orientation of aquatic invertebrates when transferred to dry land, performed by Weiss (1914) and Turner (1924), among others, indicate that in all cases when the animals have returned to the water in numbers surpassing the chance expectation, this has been due to optical stimuli. Thus the reactions of aquatic invertebrates to the humidity of the air seem never to have been tested by methods commonly used in the study of humidity reactions in terrestrial animals. The evidence about the orientation of aquatic invertebrates towards water, in fact, strongly suggests the guidance by optical stimuli only, and not by humidity gradients in the air.

Those aquatic invertebrates which *a priori* could be thought to exhibit humidity reactions are those (1) which inhabit shallow pools which may dry up during warm, rainless periods, and (2) which are considered to be either ancestors or descendants of terrestrial forms. Terrestrial animals have been evolved from aquatic ancestors. As the capacity for behavioral regulation of the body water content has been found to occur in all terrestrial forms studied, it is possible that the capacity for humidity reactions already existed in their last aquatic ancestors. Especially as the first terrestrial animals were apparently less well adapted to encounter the low humidities often locally found in the atmosphere, the occurrence of humidity reactions in these pioneers of the dry land would have rendered them a great selective advantage. In addition, humidity reactions can only be found in those aquatic animals, (3) which are able to live for some time even when exposed to the air. This condition, as well as the ability to move even when subjected to terrestrial conditions, is, of course, necessary also for the demonstration of the humidity reactions by the current experimental methods.

The above conditions are satisfied by certain aquatic members of the crustacean groups Isopoda and Amphipoda. In both of these groups terrestrial forms have

evolved separately from aquatic ancestors, and the morphological and physiological similarities between the recent aquatic and terrestrial species are greater than in most other groups with both aquatic and terrestrial species.

The test animal chosen was an aquatic isopod, *Asellus aquaticus*. It is an inhabitant of fresh water in the littoral zone of lakes and occurs in especially dense populations in shallow pools and ponds. It is also a common inhabitant of the *Fucus vesiculosus* zone in the brackish water of the Baltic and is found to occur there up to a salinity of 8‰ (records of *Asellus* exist even from 15‰). Phylogenetically, the Asellota are considered primitive. According to some observations, they are able to survive in the mud bottom of pools which have dried up (Franz, 1924, p. 467). Turner (1924) does not give any quantitive data, but states (p. 53) that "the movements of neither pond snails, asellids nor dragonfly larvae are influenced by nearby bodies of water."

For the sake of comparison, another aquatic isopod, *Idotea baltica*, was used in some experiments. This is a typical brackish-water animal, which occurs in numbers among the *Fucus* vegetation. All members of the Idoteidae are marine or brackish-water forms.

Material and Methods

The specimens of *Asellus aquaticus* were caught either from fresh-water pools in the parks of the city of Turku, SW Finland, or from brackish water (salinity about 6‰ in the vicinity of the Lohm Marine Biological Station, University of Turku) in the archipelago about 40 kilometers southeast from the city of Turku. From the latter locality, specimens of *Idotea baltica* were also collected. The animals were kept in their native water in polyethylene or glass containers at room temperature (19–21° C.), at which all experiments were also performed.

For the study of humidity reactions, the alternative chamber method of Gunn and Kennedy (1936), as modified by Perttunen (1953), was adopted. In order to obtain different relative humidities in the air of the two halves of the alternative chamber, dried silica gel with a moisture indicator was placed on one side and water on the other. The theoretical difference in the R.H.s between the sides would thus be 0–100%; the R.H.s measured by Edney paper hygrometers placed on the two sides on the false floor of brass wire gauze were about 40% and 99%, respectively. The procedure followed was otherwise that described by Perttunen (1953). The total number of position records was 2586.

In some experiments, single isopods were placed in the alternative chamber and their tracks and the periods of time spent in motion and on each side of the apparatus were recorded. The length of the different portions of the tracks were then measured with the aid of a map-measurer.

In order to find out the survival time in atmospheres with different R.H.s, the animals were kept in small glass vials in a desiccator over dried silica gel (R.H. 0%), or in a desiccator over a water surface. In the latter case, the inner surface of the desiccator was covered with wet filter paper strips extending to the water. Thus R.H. of 100% was obtained. No food or water was given to the animals. Ten animals of each group were weighed at intervals of one to eighteen hours. Animals which showed no spontaneous movements and failed to react to repeated tactile stimulation were regarded as dead. For some experiments, the antennae and antennulae were carefully removed with fine scissors.

Results

1. *The humidity reactions in the air*

The results of the humidity reaction experiments performed with *Asellus aquaticus* both from fresh and brackish water, with antennectomized animals as well as with *Idotea baltica*, appear in Table I. Both the fresh- and brackish-water specimens of *Asellus* reacted to the difference between the humidities of the air by spending more of the time in the humid part of the chamber. This reaction was more pronounced in the brackish-water specimens. The removal of the antennae and antennulae did not alter the outcome of the experiments. Thus the receptors involved in the humidity perception in *Asellus* seem not to be located on the antennae or antennulae.

Table I

The humidity reactions of Asellus aquaticus *and* Idotea baltica

	Number of test animals	Distribution of position records	Chi square	p	Intensity of reaction
Asellus aquaticus					
Fresh-water specimens:					
Intact	150	W* 285 D* 165	32.0	<0.0005	+26.7%
Antennectomized	80	W 205 D 115	25.3	<0.0005	+28.1%
Brackish-water specimens:					
Intact	150	W 413 D 187	84.4	<0.0005	+37.5%
Antennectomized	154	W 408 D 208	64.3	<0.0005	+32.3%
Idotea baltica	150	W 315 D 285	1.5	<0.2	+ 5.0%

* W, position records on the moist side; D, position records on the dry side.

Further attempts to locate the humidity receptors were made by removal of one pair of thoracic appendages and by removal of the pleopods. These operations always resulted in disturbance of the movements of the animals and the removal of the pleopods finally caused their death. Thus, by this method, no positive results concerning the location of the humidity receptors could be obtained.

The other species studied, *Idotea baltica*, did not show any definite reaction to the humidity of the air. The distribution of the animals in the alternative chamber was not found to deviate significantly from the chance expectation.

2. *The orientation types involved in the humidity reaction*

Tracks followed by 10 specimens of *Asellus* during 10 minutes in the alternative chamber were analyzed. The time spent in the dry side of the chamber was an average of 1.8 minutes (variation from 0 to 4 minutes), while in the wet side of the

chamber 8.2 minutes were spent as an average by the test animals (variation from 6 to 10 minutes). When approaching the boundary between the sides of the apparatus, the animals sometimes turned through an angle of 180° and then moved back to the side from which they had approached the boundary. This happened in 9 cases when the animal was coming to the boundary from the humid side, and never when the animal was coming to the boundary from the dry side of the chamber. Thus the klinotactic orientation mechanism (Fraenkel and Gunn, 1940) seemed to play a role in the humidity reactions of *Asellus*.

The mean velocities of the movement of the animals counted for each side of the chamber were the following: 18.1 cm. per minute in the dry and 7. 9 cm. per

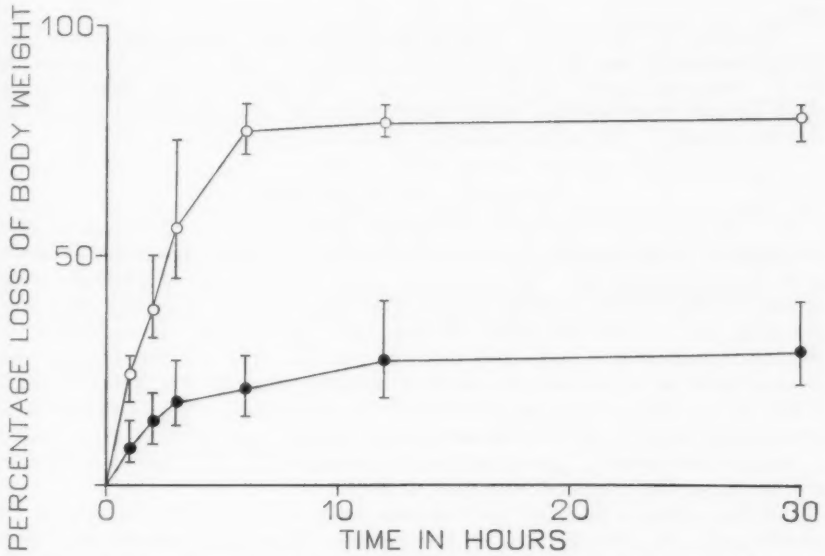


FIGURE 1. Percentage loss of body weight of *Asellus aquaticus* in dry atmosphere (upper graph) and in atmosphere saturated with water vapor (lower graph) at 20° C. Variation range is shown at each mean.

minute in the humid side. The mean number of stops per minute was on the dry side 1.2 and on the humid side 1.4. The differences between these values were not statistically significant. The mean number of random turnings per minute was 4.0 in the dry and 0.8 in the humid side of the apparatus. Owing to the large variation in the individual scores, even this difference was not statistically secured (p between 0.2 and 0.1). Although these results would suggest the unimportance of the orthokinetic and klinokinetic orientation mechanisms in the humidity reactions of *Asellus*, the tendency for orthokinesis and klinokinesis appearing from the results was especially strong in those individuals which failed to show klinotactic behavior.

3. The survival time and the rate of water loss in the air

When specimens of *Asellus* (mean initial weight 22 mg.) were subjected to dry atmosphere in a desiccator containing dried silica gel, all animals in less than one hour became unable to regain their normal position if turned on their backs. In one hour the animals had lost an average of 24% of their body weight (Fig. 1). Fifty per cent of the animals died in less than two hours (mean weight loss 38%), and not one survived for longer than four hours. Of those animals (mean initial weight 22 mg.) subjected to a saturated atmosphere in a desiccator containing water, 50% lived for more than 30 hours (mean weight loss 20%), and one for more than three days. Thus greater humidity of the air definitely enhances the survival of *Asellus* when out of water, by decreasing the rate of water loss.

According to Palmén and Suomalainen (1945), the mean lethal weight loss during desiccation was 34 to 43% for the terrestrial isopod, *Porcellio scaber*, at 20° C. This was attained in 12 to 18 hours. The present results show that the lethal amount of water loss during desiccation was approximately the same in *Asellus aquaticus*. The water content of *Asellus* was about 80%.

Discussion and Conclusions

In addition to the amphibious vertebrate, *Triton* (Czeloth, 1930), an aquatic invertebrate, *Asellus aquaticus*, has now been shown to exhibit behavioral reactions to the humidity of the air. When exposed to air, the selecting of a humid habitat apparently greatly increases the possiblities of survival in this species by decreasing the water loss. Such behavioral reactions have certainly been advantageous for the first terrestrial and amphibious forms of isopods. The fact that the antennae or antennulae do not contain the receptors mediating the response to the humidity of the air, in addition to the fact that the salinity receptors of this species have been shown to be mainly located on the antennae (Lagerspetz and Mattila, 1961), seems to indicate that humidity perception is not a modified form of salinity perception in this animal, but a separate receptive capacity.

Further attempts to locate the humidity receptors were futile because of the trouble caused by the operation to the animals. Humidity reactions have been earlier found to occur in terrestrial isopods by Gunn (1937) and by Waloff (1941). However, Gunn (1937) failed to demonstrate any humidity receptors in wood-lice. Gunn suggested that the receptors probably lie in the thoracic region. Waloff (1941) also pointed out the possibility that the concentration of body fluids in a dry atmosphere would in itself have an effect on the reactions of the animals through proprioceptors. A detailed electrophysiological study of the external receptors and proprioceptors of isopods would probably be necessary in order to gather further information on this point.

The humidity orientation mechanisms of terrestrial isopods have been studied closely by Gunn (1937), who considered the orthokinetic orientation to be the most important orientation mechanism in the humidity reactions of isopods, and by Waloff (1941), who found both orthokinesis and klinokinesis to be of importance in the humidity orientation of terrestrial isopods. The present results suggest that also the klinotactic type of orientation is important for the selection of humid habitats by *Asellus*, when taken out of water.

LITERATURE CITED

CZELOTH, H., 1930. Raumorientierung von *Triton*. *Zeitschr. vergl. Physiol.*, **13**: 74–163.

FRAENKEL, G. S., AND D. L. GUNN, 1940. The Orientation of Animals. Oxford University Press, Oxford.

FRANZ, V., 1924. Geschichte der Organismen. Gustav Fischer, Jena.

GUNN, D. L., 1937. The humidity reactions of the wood-louse, *Porcellio scaber*. *J. Exp. Biol.*, **14**: 178–186.

GUNN, D. L., AND J. S. KENNEDY, 1936. Apparatus for investigating the reactions of land arthropods to humidity. *J. Exp. Biol.*, **13**: 450–459.

HEIMBURGER, H. V., 1924. Reactions of earthworms to temperature and atmospheric humidity. *Ecology*, **5**: 276–282.

LAGERSPETZ, K., AND E. JÄYNÄS, 1959. The behavioural regulation of the water content in *Linyphia montana* (Aran., Linyphiidae) and some other spiders. *Ann. Entomol. Fenn.*, **25**: 210–223.

LAGERSPETZ, K., AND MAIJA MATTILA, 1961. Salinity reactions of some fresh- and brackish-water crustaceans. *Biol. Bull.*, **120**: 44–53.

MAXWELL, S. S., 1897. Beiträge zur Gehirnphysiologie der Anneliden. *Pflüger's Arch.*, **67**: 263–297.

PALMÉN, E., AND H. SUOMALAINEN, 1945. Experimentelle Untersuchungen über die Transpiration bei einigen Arthropoden, insbesondere Käfern. *Ann. Zool. Soc. Vanamo*, **11**, 2: 1–52.

PERTTUNEN, V., 1953. Reactions of diplopods to the relative humidity of the air. *Ann. Zool. Soc. Vanamo*, **16**, 1: 1–69.

TURNER, C. H., 1924. A new field method of investigating the hydrotropisms of fresh-water invertebrates. *Biol. Bull.*, **46**: 35–54.

WALOFF, N., 1941. The mechanisms of humidity reactions of terrestrial isopods. *J. Exp. Biol.*, **18**: 115–135.

WEISS, H. B., 1914. Notes on the positive hydrotropisms of *Gerris marginatus* Say and *Dineutes assimilis* Aubé. *Canad. Entomologist*, **46**: 33–34.

SALINITY REACTIONS OF SOME FRESH- AND BRACKISH-WATER CRUSTACEANS

KARI LAGERSPETZ AND MAIJA MATTILA

Department of Zoology and Lohm Marine Biological Station, University of Turku, Turku, Finland

Knowledge of the behavioral reactions of aquatic invertebrates to chemical stimuli has been reviewed by Warden, Jenkins and Warner (1940) and, more recently, by Hodgson (1955). The reaction thresholds of various invertebrate animals have been determined for various substances, especially for those known to stimulate the vertebrate chemoreceptors, *e.g.*, for several salts and alcohols. In addition, the part played by chemoreception in food recognition has been amply documented. The methods of study have been based either on the use of alternative chambers and gradient apparatus or on the conditioning of the behavior of the animals. In addition, the physiology of the chemoreceptors in some aquatic invertebrates has recently been studied by electrophysiological means (*Callinectes*, Hodgson, Lettwin and Roeder, 1955; *Limulus*, Barber, 1956; *Cambarus*, Hodgson, 1958).

The major part of the Baltic has a low and stable salinity. However, along the coasts of the northern Baltic, there occur periods of extreme dilution of the surface water, owing to the melting of snow and ice and the increased fresh-water output of the rivers in the spring. In such conditions, the capacity of selecting appropriate salinities could have a survival value for the brackish-water animals.

The behavioral discrimination of various salinities has been demonstrated in three semi-terrestrial crustaceans, *Ligia baudiniana* (Barnes, 1935, 1938, 1940), *Birgus latro* (Gross, 1955), and *Pachygrapsus crassipes* (Gross, 1957). In addition, Krijgsman and Krijgsman (1954) have found the South-African rock lobster, *Jasus lalandii*, to be capable of osmoreception. Earlier observations of Giersberg (1926) on *Octopus vulgaris* indicate that the reaction thresholds for salinity are in this species too high to allow the behavioral reactions to occur in natural conditions. Spiegel (1927) found that *Crangon vulgaris* reacted to higher concentrations of sea water than those found in its natural habitat.

As only a few studies dealing with the reactions of aquatic invertebrates to differences in the concentration of natural sea water were available, the salinity reactions of some fresh- and brackish-water crustaceans were studied by using an alternative chamber suitable for aquatic animals. The apparatus of Hodgson (1951) was adopted as the experimental device.

The following problems were studied: (1) Do the animals behaviorally discriminate between waters of different salinities? (2) Which are the concentrations preferred? (3) Are the reaction thresholds low enough to allow the reactions to play any part in the natural orientation of the animals? Additional information was sought on the following points: (4) Are the reactions to pure

NaCl solutions similar to those to diluted natural sea water? (5) Where are the receptors mediating the salinity reactions placed?

As test animals, the following crustaceans were used: *Asellus aquaticus* (Isopoda) both from fresh and brackish water, *Idotea baltica* (Isopoda) from brackish water, and *Gammarus* spp. (Amphipoda) from brackish water. It appeared that of the specimens of *Gammarus* used in experiments, 88% belonged to the species *G. oceanicus*, 7% to *G. locusta*, 3% to *G. zaddachi*, and 2% to *G. salinus*.

Material and Methods

For the experiments with fresh-water *Asellus*, the animals were caught in two shallow ponds in the parks of the city of Turku, southwestern Finland, in October, 1958, and in May and October, 1959. The brackish-water specimens of *Asellus* were collected in June, 1959, together with specimens of *Idotea* and *Gammarus* from the *Fucus vesiculosus* vegetation at Lohm Marine Biological Station in the Finnish southwestern archipelago, about 40 km. southwest from Turku. The experiments with brackish-water animals were performed at the Lohm Station. In the laboratory, the test animals were kept in their native, aerated water in polyethylene containers at room temperature (19–21° C.).

The apparatus of Hodgson (1951) was only slightly modified, *i.e.*, the water flowing to the funnels D in Hodgson's Figure 1 came directly from two separate storage bottles containing the solutions in question. Care was taken to have the temperatures of the two solutions equal. In some preliminary experiments, water at different temperatures was allowed to flow through the sides of the reaction tube. When the difference between the temperatures was less than 5° C., the distribution of the animals in the apparatus did not deviate from the chance expectation. In the salinity reaction experiments, the temperature difference was always less than 0.1° C. The pH of the solutions was also controlled. Groups of 10 or 20 specimens were transferred to the alternative chamber through its left end, or, in every second experiment, through its right end. The number of animals on each side of the chamber was recorded at intervals of two minutes. At intervals of 10 minutes, the tubing screws of the apparatus were adjusted so that the solutions changed sides. The mean flow rate from the reaction tube to the sink was 200 ml. per minute. The duration of the experiment was 40 to 60 minutes. All experiments were performed at room temperature (19–21° C.). The control experiments with stained solutions showed that the solutions mixed only in a 0.5 cm. zone in the middle of the chamber. After the change of the sides, the distinct boundary was re-established in one minute. When one solution at the same temperature was allowed to flow through both halves of the chamber, the test animals showed no preference for either side. Except for specific purposes, the animals were used only once for the experiments. Altogether, 1290 test animals were used and their positions in the reaction tube were recorded 26,950 times.

Results

1. *Experiments with Asellus from fresh water*

From the results of the experimental series 1, presented in Table I, it appears that the test animals were distributed at random in the chamber when the alternatives were tap water and Baltic brackish water with a salinity of 5.4‰.

The reaction threshold for pure NaCl was more closely studied, using fresh-water *Asellus*. The results of these experiments (series 2–6) appear in Table I. There are several ways of defining the threshold values, the most common being the intensity of the stimulus which evokes a response 50% of the times it is applied. Thus, if the animals in question have, through chemoreception and appropriate orientation mechanisms, selected one of the solutions in 50% of the cases when they have approached the boundary of the solutions, and the other 50% of the animals are distributed at random between the two solutions, 75% of the

TABLE I

Salinity reactions of Asellus aquaticus *from fresh water*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
1	Tap water 5.4‰ B.W.*	30	311 289	0.8	0.4
2	Tap water 15‰ NaCl	60	944 256	394	<0.0005
3	Tap water 10‰ NaCl	60	908 292	316	<0.0005
4	Tap water 5‰ NaCl	60	814 386	153	<0.0005
5	Tap water 2‰ NaCl	30	346 256	14.1	<0.0005
6	Tap water 1‰ NaCl	30	477 273	55.5	<0.0005
7	5‰ NaCl 15‰ NaCl	60	831 369	178	<0.0005
8	5‰ NaCl 10‰ NaCl	60	679 503	31.2	<0.0005
9	10‰ NaCl 15‰ NaCl	60	700 500	33.3	<0.0005

* B.W., brackish water.

animals should be on one side and 25% on the other side of the boundary, when the threshold value for the reaction is reached. Another way of measuring the capacity for discriminative behavior is to determine the upper limit for the zone of indifferent reaction (U.L.I.R.). This is the maximal value of the stimulus which does not evoke a statistically significant response. The reaction threshold of fresh-water *Asellus* for NaCl was about 10‰, while the U.L.I.R. for tap water and NaCl was below 1‰.

The differential reaction threshold for pure NaCl was also studied with

TABLE II

Effect of antennectomy on the salinity reaction of Asellus aquaticus *from fresh water*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
10	Tap water 15‰ NaCl	60 antennectomized	566 634	3.85	<0.05
11	Tap water 15‰ NaCl	60 antennectomized after 1–3 days	673 527	17.8	<0.0005

Asellus. The results of series 7–9 are given in Table I. The differential reaction threshold is defined as the difference of the stimulus values for which 50% of the animals show reaction, the others being distributed at random. The differential reaction threshold on both sides of 10‰ NaCl is more than 10‰ (*i.e.*, higher than from 5 to 15‰). In these, as in the previous cases, specimens of fresh-water *Asellus* preferred the lower concentration alternative.

The position of the receptors responsible for the reaction to NaCl solutions was studied by recording the reactions of antennectomized specimens of *Asellus*. Series 10 was performed immediately after the removal of the antennae and antennulae and series 11 one to three days later with the same animals (Table II). When compared with the data from series 2, presented in Table I, it appears that antennectomy destroyed the reaction to NaCl. Thus, the chemoreceptors mediating this reaction are apparently situated for the most part on the antennae and/or antennulae in *Asellus*.

2. *Experiments with Asellus from brackish water*

The results of the experiments with brackish-water specimens of *Asellus* are presented in Table III. The results of series 12 show that even the specimens

TABLE III

Salinity reactions of Asellus aquaticus *from brackish water*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
12	Tap water 6‰ B.W.	60	585 615	0.75	0.4
13	Tap water 10‰ NaCl	60	820 380	161.4	<0.0005
14	Tap water 5‰ NaCl	60	705 495	36.7	<0.0005
15	Tap water 1‰ NaCl	60	592 608	0.2	0.7

of *Asellus* living in the brackish water do not discriminate between tap water and their native 6‰ brackish water.

In order to determine the reaction threshold for pure NaCl solutions in brackish-water *Asellus*, experimental series 13–15 were performed. It was found that the reaction threshold is above 10‰, the U.L.I.R. for tap water and NaCl being about 1‰.

3. *Experiments with Idotea baltica*

For the sake of comparison, the reactions of a typical brackish-water isopod, *Idotea baltica*, were tested in tap water *versus* its native 6‰ brackish water. Sixty test animals were used. The animals were recorded 476 times on the side with fresh water and 724 times on the side with brackish water, the difference being significant (chi square 51.2, $p < 0.0005$).

4. *Experiments with Gammarus spp.*

As the bulk (88%) of the test animals belonged to the species *Gammarus oceanicus*, the results given here will probably mostly reflect the chemoreceptory conditions in this species. The results are presented in Table IV. *Gammarus* was the only animal used in this study which strongly reacted to the difference between tap water and 6‰ brackish water (series 17). As its preference for the brackish water seemed to be pronounced, its reactions to smaller salinity differences were tested for brackish waters with salinities of 5 *versus* 6‰ (series 18). The result, however, did not differ significantly from the chance expectation.

TABLE IV

Salinity reactions of Gammarus *spp.*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
17	Tap water 6‰ B.W.	60	109 1091	804	<0.0005
18	5‰ B.W. 6‰ B.W.	60	577 623	1.76	0.2
19	Tap water 6‰ B.W.	60 without antennulae	320 880	261	<0.0005
20	Tap water 6‰ B,W,	60 without antennae	216 984	492	<0.0005
21	Tap water 6‰ B.W.	60 without antennae and antennulae	598 602	0.01	>0.9
22	Tap water 6‰ NaCl	60	546 654	9.7	<0.01
23	Tap water 6‰ NaCl	60 without antennae and antennulae	597 603	0.03	>0.9

In order to find the position of the chemoreceptors involved in the salinity perception in these amphipods, the antennulae were removed from 60 animals and the reactions of the animals to the difference between tap water and 6‰ brackish water were tested (series 19). When compared with the results of series 17, it was found that the intensity of the reaction was reduced. The intensity of the reaction can be calculated by the following formula:

$$\frac{A - B}{N} \cdot 100 = R.$$

If A and B represent the numbers of records of animals on each side of the reaction tube and N is the total number of position records, then R is the excess percentage of records on the side with A animals. The intensities of reaction measured as the excess percentages on the side with 6‰ brackish water, calculated for the experimental series 17 and 19–21, were as follows:

17: 6‰ brackish water *vs.* tap water, normal animals	+81.8%
19: as above, antennulae removed	+46.7%
20: as above, antennae removed	+64.0%
21: as above, antennulae and antennae removed	+ 0.3%
Chance expectation	0.0%

Thus, the removal of both antennae and antennulae totally abolished the salinity reactions in *Gammarus*. The removal of antennulae, only, reduced the intensity of the salinity reaction, as did the removal of the antennae, although to a lesser extent. It seems safe to assume that *Gammarus* has the chemoreceptors responsible for orientation in a salinity gradient on its pairs of antennulae and antennae, the antennulae being apparently somewhat more important in salinity perception.

It seemed worthwhile to explore whether *Gammarus,* which preferred brackish water with a salinity of 6‰ (expressed as NaCl), would react similarly to pure 6‰ NaCl solutions. Thus, the experiments of series 22 (Table IV) were performed. The intensity of the reaction was much weaker than for 6‰ brackish water, but still positive for the saline medium (excess percentage + 9.0%). The removal of antennulae and antennae (series 23) also in this case abolished the salinity preference reaction, the excess percentage being reduced to 0.5%. The receptors involved in the chemoreception of pure NaCl are thus also situated on the antennulae and/or antennae.

Discussion

1. *The significance of salinity reactions in nature*

It was shown that the specimens of *Asellus* from fresh and from brackish water did not behaviorally react to the difference between tap water and Baltic brackish water with a salinity of 5.4‰ or 6‰. These salinities are approximately those which usually prevail in the natural habitats of *Asellus aquaticus* in wide areas of the northern Baltic. As no reaction occurs for this large difference, the com-

pensation for the much smaller salinity variations (occurring, for example, on the shores as a consequence of the melting of the ice in spring) by movement of the animals to other habitats seems utterly improbable.

Specimens of *Gammarus* clearly reacted to the difference between fresh water and 6‰ brackish water, but they did not show a significant response to the difference between 5‰ and 6‰ brackish water. The reaction of *Idotea baltica* to the difference between fresh water and 6‰ brackish water was even less pronounced than that of *Gammarus*. Thus, the adaptive value of behavioral reactions to differences in salinity in the crustaceans studied does not seem to have been established by the results of this study. However, the methods of investigation may be criticized for not allowing longer times than 10 minutes for the discriminative reactions to the alternatives studied. However, in several cases with pure NaCl solutions, this period of time was found to be entirely sufficient for the performance of definite responses by most of the animals. The possible significance of behavioral salinity reaction mechanisms in other truly aquatic brackish-water animals, for example in estuarine forms, deserves further attention.

2. *The salinity reaction thresholds*

Most of the experimental series were devoted to the determination of salinity reaction thresholds. It appeared that the reaction threshold for NaCl is somewhat higher for the brackish-water specimens of *Asellus* than for the fresh-water ones. Similarly, the U.L.I.R. between fresh water and NaCl is higher in the brackish-water specimens. It would be interesting to find out whether this is a result of a sensory or some other physiological adaptation process, or an indication of physiological race formation. As reported previously (Lagerspetz, 1958), and afterwards confirmed by repeated tests, the brackish-water specimens of *Asellus* do not survive a longer stay in fresh water, while the fresh-water specimens are apparently not injured by a transfer to Baltic brackish water. Thus, one could at least study the effects of the stay of the fresh-water specimens in brackish water on their salinity reaction threshold.

The experiments for the determination of the differential salinity reaction threshold in *Asellus* showed that these animals are not only capable of discriminating fresh water from saline solutions, but also react differently to different concentrations of NaCl.

Fresh water or the weaker NaCl solution was preferred by *Asellus*, which showed no significant reaction to the brackish water. However, the brackish-water specimens were rather more often found on the side with brackish water, and the fresh-water animals slightly more often on the side with fresh water. In all cases, the number of animals on the brackish-water side was significantly higher for brackish-water animals. *Gammarus* slightly but significantly preferred 6‰ NaCl to fresh water. Such a comparatively strong NaCl solution was very definitely rejected by fresh-water *Asellus*, but also to a lesser degree by the brackish-water specimens. The reaction of *Gammarus* in brackish-water *versus* fresh-water experiments was again strongly positive towards brackish water. Thus, the following modifications may be linked with the progressive adaptation of fresh-water animals to brackish water:

(1) The rise of the NaCl reaction threshold by the development of a slight preference for dilute solutions of NaCl to fresh water.

(2) The development of a strong preference for brackish water to fresh water.

3. *The location of chemoreceptors*

The location of chemoreceptors in Crustacea has been documented and discussed in some twenty papers since 1887. Most experiments hitherto made have been performed on Decapoda. Among the exceptions are the early observations of May (1887) on *Mysis flexuosa* (Mysidacea), studies by Abraham and Wolsky (1930) on land isopods, by Uchida (1930) on the sex recognition in *Asellus,* and by Seifert (1930) on *Triops cancriformis* (Branchiopoda).

The chemoreceptors may be specialized to mediate responses only for certain chemical substances. Thus, the different receptor types may also have different sites in the animal. The results of experiments with one or a few substances cannot thus be generally applied to the receptors for other substances. It is therefore understandable that different authors have attained different results, even when working with the same species. In decapods, antennulae, antennae, mouth parts, tips of the appendages and the entire body surface have been variously described as the sites of chemoreceptors. Most of the evidence supports the special importance of the external ramus of the antennulae for the "distance" chemoreception, and of the mouth parts and other appendages for the contact chemoreception. The role of the receptors on the antennulae of *Callinectes* and *Cambarus* in chemoreception has also been proved through the electrophysiological studies of Hodgson, Lettwin and Roeder (1955) and Hodgson (1958).

As the removal of the antennae and antennulae in *Asellus* at first apparently abolished the reaction to NaCl, the chemoreceptors mediating the response seem generally to be situated on the antennae and/or antennulae. However, the results obtained for experiments performed one to three days after the amputation of the antennae and antennulae show that some sensitivity to NaCl may still exist in the animals or is rapidly regained.

More clear-cut results were obtained in the experiments with *Gammarus.* In these, the salinity receptors involved in reactions to brackish water are apparently on the antennulae and antennae. The removal of these also abolished the reaction to pure NaCl solutions. Thus, in amphipods the antennulae and antennae, and in isopods the antennulae and/or antennae, seem to contain the salinity receptors, and the results thus corroborate the various earlier observations on the location of the "distance" chemoreceptors in Crustacea.

It is tempting to think that "on terrestrial organisms the humidity has probably similar physiological effects as the salinity on aquatic organisms" (Kinne, 1957, p. 90). However, the problem faced by aquatic animals seems to be more in the maintenance of the proper ionic composition of their body fluids, while in terrestrial animals it is in the maintenance of a proper degree of dilution of the body fluids, already controlled for their ionic equilibrium. A more specific argument emerges from the present study: in *Asellus* the site of the salinity receptors is on the antennae and/or antennulae, which have recently been shown to be insignificant for the orientation of *Asellus* in an alternative chamber with different humidities of the air (Lagerspetz and Lehtonen, 1961).

The authors wish to express their indebtedness to Professors Paavo Voipio and Hannu Suomalainen (Turku) for working facilities, and to Professor J. M. von Wright (Turku) for valuable discussions.

Summary

1. The salinity reactions of *Asellus aquaticus* from fresh and Baltic brackish water (6‰), of *Idotea baltica* and of *Gammarus* spp. from brackish water were studied with the apparatus of Hodgson (1951). Both natural brackish-water and pure NaCl solutions were used.

2. None of the experimental animals had, for brackish water, reaction thresholds low enough to allow behavioral selection of salinities to occur in natural conditions.

3. The reaction threshold for NaCl solutions was higher in brackish-water than in fresh-water specimens of *Asellus*. *Asellus* always preferred the more dilute concentration. *Idotea baltica* and *Gammarus* preferred brackish water to fresh water. *Gammarus* preferred even 6‰ NaCl to fresh water.

4. In *Asellus*, the chemoreceptors mediating the response to salinity variations seem, for the most part, to be situated on the antennae and/or antennulae. In *Gammarus*, the salinity receptors are situated on the antennae and antennulae.

Literature Cited

Abraham, A., and A. Wolsky, 1930. Die Geruchsorgane der Landisopoden. *Zeitschr. Morphol. Ökol.*, **17**: 441–463.

Barber, S. B., 1956. Chemoreception and proprioception in *Limulus*. *J. Exp. Zool.*, **131**: 51–73.

Barnes, T. C., 1935. Salt requirements and orientation of *Ligia* in Bermuda. III. *Biol. Bull.*, **66**: 259–268.

Barnes, T. C., 1938. Experiments on *Ligia* in Bermuda. V. Further effects of salts of heavy sea water. *Biol. Bull.*, **74**: 108–116.

Barnes, T. C., 1940. Experiments on *Ligia* in Bermuda. VII. Further effects of sodium and magnesium. *Biol. Bull.*, **78**: 35–41.

Giersberg, H., 1926. Über den chemischen Sinn von *Octopus vulgaris* Fabr. *Zeitschr. vergl. Physiol.*, **3**: 827–838.

Gross, W. J., 1955. Aspects of osmotic regulation in crabs showing the terrestrial habit. *Amer. Nat.*, **89**: 205–222.

Gross, W. J., 1957. A behavioral mechanism for osmotic regulation in a semi-terrestrial crab. *Biol. Bull.*, **113**: 268–274.

Hodgson, E. S., 1951. Reaction thresholds of an aquatic beetle, *Laccophilus maculosus* Germ., to salts and alcohols. *Physiol. Zoöl.*, **24**: 131–140.

Hodgson, E. S., 1955. Problems in invertebrate chemoreception. *Quart. Rev. Biol.*, **30**: 331–347.

Hodgson, E. S., 1958. Electrophysiological studies of arthropod chemoreception. III. Chemoreceptors of terrestrial and fresh-water arthropods. *Biol. Bull.*, **115**: 114–125.

Hodgson, E. S., J. Y. Lettwin and K. D. Roeder, 1955. The physiology of a primary chemoreceptor unit. *Science*, **122**: 417–418.

Kinne, O., 1957. A programmatic study of comparative biology of marine and brackish-water animals. *Année Biol.*, **33**: 87–92.

Krijgsman, B. J., and N. Krijgsman, 1954. Osmorezeption in *Jasus lalandii*. *Zeitschr. vergl. Physiol.*, **37**: 78–81.

Lagerspetz, K., 1958. The brackish-water tolerance of some freshwater crustaceans. *Verh. internat. Ver. Limnol.*, **13**: 718–721.

Lagerspetz, K., and Aila Lehtonen, 1961. Humidity reactions of some aquatic isopods in the air. *Biol. Bull.*, **120**: 38–43.

MAY, K., 1887. Über das Geruchsvermögen der Krebse nebst einer Hypothese über die analytische Tätigkeit der Riechhärchen. Diss. Kiel.

SEIFERT, R., 1930. Sinnesphysiologische Untersuchungen am Keimfuss (*Triops cancriformis* Bosc.). *Zeitschr. vergl. Physiol.*, **11**: 386–436.

SPIEGEL, A., 1927. Über die Chemorezeption von *Crangon vulgaris* Fabr. *Zeitschr. vergl. Physiol.*, **6**: 688–730.

UCHIDA, T., 1930. Observations sur le sens de la reconnaisance du sexe chez *Asellus*. *Bull. Soc. Zool. France*, **55**: 516–520.

WARDEN, C. J., T. N. JENKINS AND L. H. WARNER, 1940. Comparative Psychology. Vol. II. Plants and Invertebrates. The Ronald Press, New York.

A COMPARISON OF THE RESPONSES OF TRITURUS AND DESMOGNATHUS TO THYROID-STIMULATING HORMONE ADMINISTRATION [1]

W. GARDNER LYNN [2] AND JAMES NORMAN DENT [3]

Biology Division, Oak Ridge National Laboratory,[4] Oak Ridge, Tennessee, Department of Biology, Catholic University of America, Washington 17, D. C., and University of Virginia, Charlottesville, Virginia

We have recently reported experiments comparing the effects of goitrogen treatment on the histology and the concentration of radioiodine by the thyroid glands of *Triturus viridescens* and *Desmognathus fuscus* (Dent and Lynn, 1958). The results indicate that, although these drugs markedly inhibit thyroidal concentration of iodine in both urodeles, characteristic histological changes in the gland occur in *Desmognathus* only. It was suggested that the failure of the *Triturus* thyroid to give the usual histological response to such treatment might be accounted for by either (1) some defect in the pituitary control of thyroid function, such that decreased thyroid hormone level in the blood does not cause any marked increase in TSH (thyroid-stimulating hormone) production, or (2) a lack of ability of the *Triturus* thyroid to respond to TSH stimulation. The present experiments were undertaken to test the latter alternative by study of the effects of administration of exogenous TSH upon histological structure and on radioiodine uptake in the thyroids of these two animals.

Materials and Methods

Specimens of *Triturus* (*Diemyctylus*) *viridescens viridescens* (Rafinesque) were collected from a pond near Monterey, Virginia. Specimens of *Desmognathus fuscus fuscus* (Rafinesque) were taken from a stream near Oliver Springs, Tennessee. The animals were kept in the laboratory for a minimum period of one month before being used for experiments. They were fed every other day, *Triturus* with ground beef fortified with cod liver oil and calcium phosphate, *Desmognathus* with live meal-worm larvae. Both before and during the experimental period the animals were kept in a constant temperature chamber at 23.0° ± 1.0° C.

Injections of 0.1 ml. of a solution of Armour's Thytropar (1.0 U.S.P. Unit per ml.) in 0.7% saline were made into the body cavities of the experimental animals on alternate days. The salamanders were always anesthetized in an aqueous solution of tricaine methane sulfonate (1 part in 1000) before injection.

[1] Supported in part by AEC Contract No. AT-(40-1)-2000 and by U. S. Public Health Service Grant No. A-2921.

[2] Department of Biology, The Catholic University of America.

[3] Department of Biology, University of Virginia.

[4] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

For comparative study of the effects of injected TSH on the thyroids of *Triturus* and *Desmognathus*, groups of each species were given various numbers of injections and were killed various periods of time after the cessation of injection. The thyroid regions of these animals were fixed in equal parts of Bouin's fluid and ethyleneglycolmonoethyl ether. After sectioning they were stained with Harris' hematoxylin and Ponceau de Xylidine-Orange II (Gray, 1952).

Radioiodine uptake by and release from the thyroid was followed by a standardized procedure. Sixteen mature specimens of *Desmognathus* (2.8–3.3 g. body weight) and sixteen mature specimens of *Triturus* (2.5–3.3 g. body weight) were selected and divided into groups of eight controls and eight experimental animals. All animals were given six successive injections, experimental animals receiving the TSH preparation and controls receiving 0.1 ml. of 0.7% NaCl solution. Three hours after the last of these injections, each animal was given an additional intraperitoneal injection of 0.1 ml. of 0.7% saline containing 50.0 μc./ml. of I^{131}. At

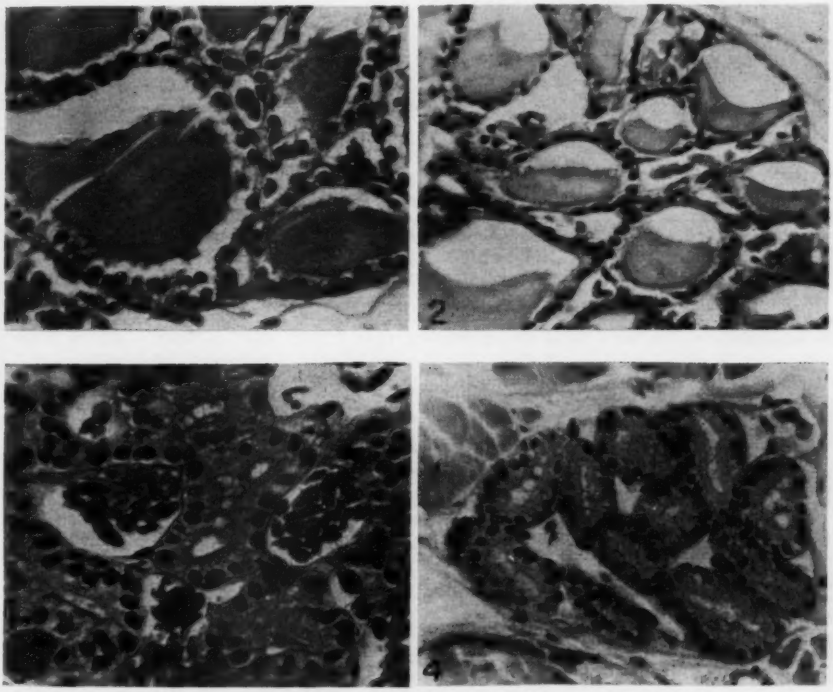


FIGURE 1. Section of thyroid gland of control specimen of *Triturus*.
FIGURE 2. Section of thyroid gland of control specimen of *Desmognathus*.
FIGURE 3. Section of thyroid gland of *Triturus* given seven successive injections of TSH on alternate days and killed 24 hours after the last injection.
FIGURE 4. Section of thyroid gland of *Desmognathus* given seven successive injections of TSH on alternate days and killed 24 hours after the last injection.

intervals of 6, 12, 24, 36, 48, and 60 hours thereafter,[5] each specimen was again anesthetized and the radioactivity of the thyroid and heart regions was measured by means of a previously developed technique (Dent and Lynn, 1958). In this technique a scintillation counter was used, consisting of a 1.5-inch NaI crystal cemented to the window of an RCA type 5819 photomultiplier tube and a conventional amplifier and binary scaler. The crystal and photomultiplier were mounted in a lead cylinder 5.2 cm. thick with a collimating slit measuring 4.0 by 12.0 mm. At the conclusion of the measurements of radioactivity, the animals were killed and their thyroids fixed and sectioned.

TABLE I

Radioactivity (corrected for physical decay) at the indicated periods of time after injection with 5 μc. of I^{131}. Experimental animals had previously received six successive injections of TSH. All means are based on observations on eight individuals.

Standard deviation: $s = \sqrt{\sum_{i}^{n} \frac{(X_i - \bar{X})^2}{n-1}}$ *where* $n = 8$.

Species	Time after I^{131} injection (hours)	Radioactivity (cts./sec.)			
		Controls		Treated	
		Thyroid	Heart	Thyroid	Heart
Triturus viridescens	6	4.43 ± 1.686	3.92 ± 1.074	5.71 ± 1.479	4.08 ± 0.856
	12	5.03 ± 1.201	3.01 ± 0.558	7.01 ± 2.224	2.74 ± 0.525
	24	4.88 ± 1.179	1.33 ± 0.694	9.41 ± 4.057	1.44 ± 0.659
	36	5.07 ± 0.962	1.16 ± 0.352	9.39 ± 3.807	1.20 ± 0.727
	48	4.93 ± 1.022	0.91 ± 0.259	9.97 ± 4.916	0.86 ± 0.493
	60	4.96 ± 1.105	0.70 ± 0.315	9.09 ± 3.805	0.90 ± 0.699
Desmognathus fuscus	6	4.29 ± 1.040	6.14 ± 1.724	7.65 ± 1.492	5.11 ± 1.726
	12	5.35 ± 1.004	4.83 ± 0.812	6.16 ± 1.731	3.66 ± 1.327
	24	4.56 ± 0.765	3.24 ± 0.702	4.44 ± 1.452	2.57 ± 0.581
	36	3.75 ± 0.645	2.10 ± 0.579	3.97 ± 1.391	1.83 ± 0.580
	48	3.26 ± 0.569	1.62 ± 0.212	3.60 ± 1.930	1.68 ± 0.311
	60	3.02 ± 0.977	1.45 ± 0.215	3.16 ± 1.431	1.42 ± 0.403

RESULTS

1. *Effects of TSH treatment on thyroid histology*

It has previously been pointed out (Dent and Lynn, 1958) that, during the summer months, the thyroids of untreated *Triturus* present a quite uniform histological appearance characteristic of relatively low activity. The follicles are well rounded, the epithelium is low, the colloid is homogeneous and acidophilic, chromophobe droplets are absent or sparse (Fig. 1). The thyroids of untreated *Des-*

[5] In preliminary experiments, radioactivity measurements were made at hourly intervals for the first 8 hours after injection of radioiodine. It was found that the activity in the thyroid reached peak levels at 5–6 hours in *Desmognathus* but was still rising steadily in *Triturus* at 8 hours. In all later experiments the measurements were begun at 6 hours and continued for 60 hours at the intervals indicated.

mognathus, on the other hand, show more individual variation but all present an appearance of moderate to relatively high activity: cuboidal to columnar epithelium; less homogeneous, often basophilic, colloid; relatively abundant chromophobe droplets at the peripheries of the colloid masses (Fig. 2).

Administration of TSH caused marked changes in the thyroid in both. Thyroids of animals given seven injections (over a 13-day period) and fixed 24 hours after the last injection showed the maximal effects seen in this experiment and have been chosen for illustration (Figs. 3 and 4). It will be noted that, in both *Triturus* and *Desmognathus,* the epithelium increased greatly in height, the colloid was almost entirely discharged from the gland, and the follicles became irregular in shape. Study of the thyroids of animals given smaller numbers of injections

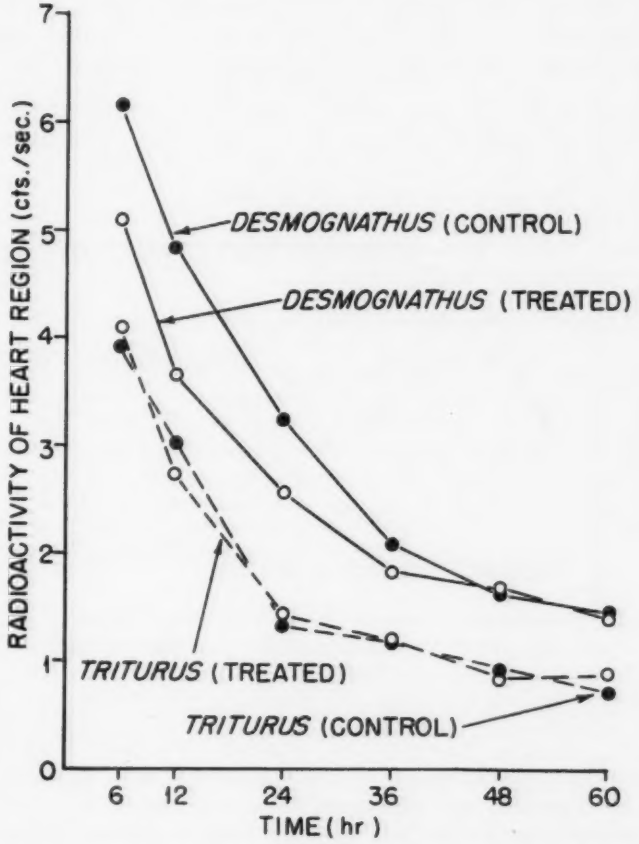


FIGURE 5. Radioactivity in the heart regions of experimental and control specimens of *Desmognathus* and *Triturus* over a period of 60 hours after administration of radioiodine.

shows that these same effects are obtained with six, five, or four injections. Thyroids of salamanders given three or two injections showed less marked but definite histological responses. Thyroids of animals that received a single injection could not be distinguished from those of controls.

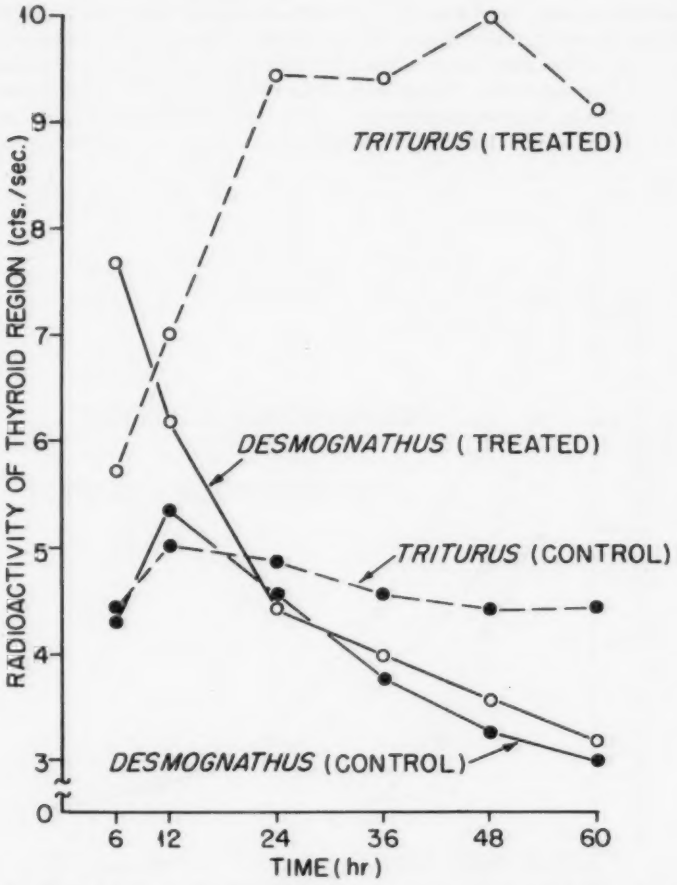


FIGURE 6. Radioactivity in the thyroid regions of experimental and control specimens of *Desmognathus* and *Triturus* over a period of 60 hours after administration of radioiodine.

By giving a sufficient number of injections of TSH to ensure marked responses (4–8 injections), and varying the times of fixation after the last injection, it was ascertained that the maximal histological response persists for 36–48 hours. Thyroids of specimens fixed 60 hours after the last TSH administration showed definite indication of regression toward the normal histology with a decrease in

epithelial height and the beginning of accumulation of stored colloid. By 75 hours regression was well under way and by 144 hours the thyroids of treated salamanders were like those of controls.

2. Effects of TSH treatment on uptake of radioiodine by the thyroid

The means of counts of radioactivity (corrected for physical decay) in thyroid and heart regions of control and experimental animals at successive periods after injection of I^{131} are shown in Table I, and these data are illustrated graphically in Figures 5 and 6.

It will be noted (Fig. 5) that the heart region, which we consider as representative of soft tissues other than those of the thyroid, shows relatively high counts at 6 hours after I^{131} administration in both control and experimental groups of both species. The counts then decline rapidly as the radioiodine is eliminated from the circulating blood. There is no significant difference between experimental and control groups within the species, but the counts for the *Triturus* heart region are uniformly lower than those for *Desmognathus*.

Counts for the thyroid region (Fig. 6) reveal that treatment with TSH results in an increased uptake of radioiodine by the gland in both *Triturus* and *Desmognathus*. At 6 hours after I^{131} injection, the counts in the thyroid regions of both groups of experimental animals are well above those in the corresponding controls. However, the later counts show important differences between the two species. In *Desmognathus*, the counts decline quickly and in 24 hours have reached the level of the control thyroids. Thus, the initial rate of uptake of radioiodine is high but the rate of turnover is also high and there is no significant retention of I^{131} in the thyroids of experimental animals as compared with those of controls after 12 hours. In *Triturus*, on the other hand, the counts rise more slowly in the period up to 24 hours after I^{131} injection and then level off with little sign of decline by the end of the experiment (60 hours). In this species, then, the TSH treatment caused increase in radioiodine uptake and this, combined with a slow rate of turnover, resulted in retention of I^{131} at a much higher level than in the controls. It may be noted that the curve for the thyroids of *Triturus* control specimens shows little change in counts throughout the counting period. This indicates the relatively low rate of activity of the thyroid in normal animals of *Triturus* which we have reported previously (Dent and Lynn, 1958).

Discussion

Our earlier experiments indicated that, although both *Triturus* and *Desmognathus* show a marked inhibition of thyroid function after treatment with goitrogenic drugs, the usual histological changes that occur after goitrogen treatment are not seen in *Triturus* during treatment periods of less than two months duration.[6] These histological changes are normally induced by an increased production of pituitary thyrotropin after a decrease in the level of circulating thyroid hormone. The absence of such changes in the *Triturus* thyroid could indicate either a failure of the pituitary to produce significantly higher levels of thyrotropin after thyroid

[6] Adams, 1946a, and Dent (unpublished) have shown that the thyroid of *Triturus* undergoes hyperplasia after several months of treatment with goitrogens.

inhibition or a failure of the thyroid to respond in the typical way to thyrotropin stimulation.

The results reported here show clearly that the *Triturus* thyroid is capable of responding to exogenous thyrotropin. The histological response of the *Triturus* thyroid proved to be entirely comparable to that of the *Desmognathus* thyroid and the effect of TSH treatment on radioiodine uptake was even more marked in the former than in the latter. These findings thus indicate that the histological signs of low thyroid activity in the normal gland of *Triturus,* and its failure to show signs of increased activity after goitrogen treatment, are to be attributed to a low level of thyrotropin production.

The fact that treatment with exogenous TSH causes such a marked increase in radioiodine uptake in *Triturus* as compared with *Desmognathus* may be taken as further evidence that TSH production is unusually low in *Triturus.* Several previous workers have concluded that the responsiveness of the thyroid to exogenous TSH varies inversely with the thyrotropin content or production of the animal's own pituitary (Loeb and Friedman, 1930; Collip and Anderson, 1935). Indeed, the selection of the young guinea pig and the chick as favorable animals for assay of thyrotropin is based on the relatively low thyrotropin content of the pituitary in these animals and the accompanying high sensitivity of their thyroids (Adams, 1946b). Among cold-blooded vertebrates the goldfish (*Carassius auratus*) is the best known example of a species in which the thyroid normally presents a histological appearance of very low activity. The goldfish thyroid, like that of *Triturus,* shows no histological evidence of increased activity after goitrogen administration (Fortune, 1955) but responds strongly to injected TSH (Gorbman, 1940; Berg and Gorbman, 1954). Fortune (1956) has suggested that in the goldfish the inactive thyroid, resulting from absence or very low level of TSH production, may be an important factor in this animal's exceptional ability to withstand high temperature. We have previously pointed out (Dent and Lynn, 1958) that this hypothesis may be valid for *Triturus* as well, since this salamander also has a high upper limit of temperature tolerance.

We wish to acknowledge the assistance of Dr. M. A. Kastenbaum of the Oak Ridge National Laboratory Mathematics Panel in the statistical analysis.

Summary

1. Specimens of *Triturus viridescens* and *Desmognathus fuscus* were given injections of Armour's Thytropar on alternate days.

2. Histological examination of the thyroid glands of experimental and control animals showed that both species gave maximal response after only four injections, the follicular epithelium being greatly heightened and intrafollicular colloid almost completely discharged, and that the response persisted for 3–4 days after cessation of treatment, but then declined and disappeared by 7 days.

3. Measurements of uptake and turnover of I^{131} by the thyroids of animals given six injections of thyrotropin showed a high uptake and a rapid turnover in *Desmognathus.* In *Triturus* the effect of the treatment upon I^{131} uptake was also

marked and the radioiodine was retained at a high level throughout the counting period (60 hours).

4. It is concluded that the *Triturus* thyroid is more responsive to exogenous TSH than is that of *Desmognathus*, and that this is related to an exceptionally low TSH content and production in the anterior pituitary of *Triturus*.

LITERATURE CITED

ADAMS, A. E., 1946a. The effects of thiourea on the thyroids of *Triturus viridescens*. *Anat. Rec.*, **94**: 532.

ADAMS, A. E., 1946b. Variations in the potency of thyrotrophic hormone of the pituitary of animals. *Quart. Rev. Biol.*, **21**: 1–32.

BERG, O. A., AND A. GORBMAN, 1954. Normal and altered thyroidal function in domesticated goldfish, *Carassius auratus*. *Proc. Soc. Exp. Biol. Med.*, **86**: 156–159.

COLLIP, J. B., AND E. M. ANDERSON, 1935. Studies on the thyrotropic hormone of the anterior pituitary. *J. Amer. Med. Assoc.*, **104**: 965–969.

DENT, J. N., AND W. G. LYNN, 1958. A comparison of the effects of goitrogens on thyroid activity in *Triturus viridescens* and *Desmognathus fuscus*. *Biol. Bull.*, **115**: 411–420.

FORTUNE, P. Y., 1955. Comparative studies of the thyroid function in teleosts of tropical and temperate habitats. *J. Exp. Biol.*, **32**: 504–513.

FORTUNE, P. Y., 1956. An inactive thyroid gland in *Carassius auratus*. *Nature, London*, **178**: 98.

GORBMAN, A., 1940. Suitability of the common goldfish for assay of thyrotropic hormone. *Proc. Soc. Exp. Biol. Med.*, **45**: 772–773.

GRAY, P., 1952. Handbook of Basic Microtechnique. Blakiston Company, Philadelphia.

LOEB, L., AND H. FRIEDMAN, 1930. Further investigations concerning the stimulating effect of anterior pituitary gland preparation on the thyroid gland. *Proc. Soc. Exp. Biol. Med.*, **28**: 209–213.

SYSTEMATICS AND DISTRIBUTION OF AN ESTUARINE ISOPOD CRUSTACEAN, CYATHURA POLITA (STIMPSON, 1855), NEW COMB., FROM THE GULF AND ATLANTIC SEABOARD OF THE UNITED STATES

MILTON A. MILLER[1] AND W. D. BURBANCK[2]

Department of Zoology, University of California, Davis, California, and Department of Biology, Emory University, Georgia

In estuaries and tidal marshes along the southern and eastern seaboard of the United States, there lives a burrowing anthurid isopod of the genus *Cyathura*. It is the only species of this genus thus far reported over a long range extending from Lake Pontchartrain, Louisiana to Chewonki Creek, Maine. It has long been misidentified as *Cyathura carinata* (Krøyer), a species originally described from Copenhagen Harbor, Denmark, and subsequently reported from many other localities in Europe, Greenland, the Mediterranean, Africa, and Asiatic Russia. In this paper, evidence will be presented to show that the eastern American form is specifically distinct from *C. carinata*, and it will be redescribed under its proper name.

The ecology and ecological distribution of our east coast *Cyathura* have been investigated by one of the present authors (Burbanck *et al.*, 1956; Burbanck and Burbanck, 1958; and Burbanck, 1959a, 1959b), while the other (M.A.M.) has been mainly concerned with the systematics and zoögeography of this and related species. The preceding statement indicates the general areas of responsibility of the authors of this article.

American material for this study was obtained from the U. S. National Museum through the courtesy of Drs. Fenner Chace and T. E. Bowman, amply supplemented by W. D. Burbanck's extensive collections of eastern American *Cyathura* from its known range. From South Africa, Dr. K. H. Barnard kindly sent paratypes of *Cyathura estuarius* Barnard (1914) which he later (1925) assigned to the synonomy of *C. carinata* (Krøyer). European material from the type locality and elsewhere was graciously supplied by Drs. Torben Wolff, A. Panning and E. Rasmussen. The authors take this opportunity to acknowledge the valuable assistance of these esteemed contributors. We also wish to thank many others whose names and assistance are mentioned in the text. Finally, we are grateful to Dr. G. Victor Morejohn for the illustrations and technical assistance.

The nomenclatorial history of the eastern American *Cyathura* has been quite confused. It was first mentioned in the literature under the name *Anthura gracilis* Montagu by Gould (1841) and later by DeKay (1844) in faunal accounts of Massachusetts and New York, respectively. Subsequently it was twice described

[1] Contribution from the Department of Zoology, University of California, Davis.

[2] Supported by National Science Foundation Grant G-7138 and by the McCandless Fund of Emory University.

as a new species—first, by Stimpson (1855) under the name *A. polita,* and nearly twenty years later by Harger (in Verrill, 1873) under the name *A. brunnea.* In 1878, however, Harger (1880) rightly relegated his species to the synonomy of Stimpson's earlier described *A. polita.* At the same time, he noted that the American forms approached the descriptions and figures of *A. carinata* Krøyer, but he also noted many discrepancies and regretted the lack of European specimens for comparison.

Although Harger did observe a resemblance between the European and American forms, he apparently did not regard them as conspecific. As a matter of record, Harger (1879) considered *Anthura polita* to be (p. 162) "A southern species, not found north of Cape Cod until the summer of 1878, when it was taken at Gloucester, Mass."

In 1886, Norman and Stebbing founded the genus *Cyathura* for *Anthura carinata* Krøyer. They formally placed *A. gracilis,* DeKay (*non* Montagu), *A. polita* Stimpson, and *A. brunnea* Harger in the synonomy of *Cyathura carinata* (Krøyer), thus establishing *Cyathura* as a monotypic genus. Unfortunately, they based the description of the new genus on American material which they mistakenly considered to be conspecific with *C. carinata.* "Our description," they state (p. 125), "is drawn from specimens kindly sent to us, named *Anthura brunnea,* by Mr. S. I. Smith, the talented carcinologist of Yale College. There can be no doubt, we think that it is the *A. carinata* of Kröyer." They dismissed Harger's mention of disagreements between American specimens and Krøyer's (1847 and 1849) description with the statement that (p. 125) ". . . if the figures of the parts so described which are given in the 'Voyage en Scandinavia' &c be examined the apparent discrepancies seem to disappear." Had careful comparisons been made by these early authors between representative European and American specimens, it is likely that the differences, which we note later, would have been observed. As it is, the key source of the subsequent confusion was their compound error, first in placing the American species in the synonomy of *A. carinata* Krøyer, the type of their new genus *Cyathura,* and then basing their genotypic description on the misidentified American specimens.

Richardson (1900, 1905) naturally followed Norman and Stebbing's disposition which until now has never been seriously questioned. In her monograph (1905), Richardson noted that three specimens—two from Florida and one from South Carolina—differed in certain respects from other specimens, but she did not regard these variations as particularly significant.

The subsequent general acceptance of the conclusion that the eastern American form is conspecific with *Cyathura carinata* (Krøyer) is not surprising in view of the eminence of the above-mentioned authorities, the misidentified "genotype," and the superficial similarity of many anthurid species, coupled with the inadequacy of early descriptions and illustrations. Moreover, a rather intriguing zoögeographical picture emerged when eastern North America was added to the previously known records of *C. carinata,* for this seemed to extend its distribution in an arc around the North Atlantic basin. Allee's (1923, p. 179) listing of it as a "north ranging species" and its reported occurrence in Greenland are in line with this notion. Indeed, the question has been raised whether this species might not even be completely circum-Atlantic or even cosmopolitan in distribution. It has been

reported from the western and southern coasts of Africa (Monod, 1925; Barnard, 1925; Day *et al.*, 1952). No cyathurans have been reported, however, from the Atlantic coasts of South America, but the possibility of their occurrence there cannot be dismissed inasmuch as the shores of that continent have been inadequately explored for isopods. Mention should be made here of a West Indian species, *C. crucis* Barnard (1925), taken from a depth of four fathoms at St. Croix, Virgin Islands, and of another Caribbean species, *C. curassavica* Stork (1940) from Curacao in the Netherland Antilles.

The larger concept of world-wide distribution of *Cyathura carinata* (K.) obviously results from additional reports of this species in the Mediterranean (Stammer, 1932; Larwood, 1940), China (Tattersall, 1921), and the Okhotsk Sea (Gurjanova, 1936), as well as many references to it in Western Europe. It has not been found, however, in the entire east Pacific. Another species, *C. munda* Menzies (1951), occurs along the California coast from Marin County to the Mexican border (Menzies and Barnard, 1959), but its distributional limits have not yet been established.

An alternative zoögeographical hypothesis suggested by Burbanck (1959b, p. 508) is that the several species of *Cyathura* in the northern hemisphere, including our eastern American forms, and the Eurasian *C. carinata*, ". . . may have arisen from a common preglacial species which had a circumpolar distribution. When the original species was subdivided once or many times by lobes of glaciers during the last ice age and driven south, speciation may have occurred. Now during the present interglacial period, populations of *C. carinata* (K.) and the North American *Cyathura* sp. may be moving north again." As evidence, Burbanck cites: (1) the absence of cyathurans in Norway; (2) reports of the appearance of *C. carinata* in Sweden in the 1930's (Löwegren, 1937; Lundstrom, 1937); (3) Harger's statement that the American form is a southern species that got only as far north as Gloucester, Mass., in 1878; and (4) the unsuccessful attempts of himself and others to find cyathurans north of Maine in the Gulf of St. Lawrence, Baie des Chaleurs, Cape Breton Island, and Mount Desert Island. Furthermore, the Greenland report must be discounted since Dr. Torben Wolff (personal communication) has been unable to find any authentic record of *C. carinata* from Greenland. It is apparently either a case of mistaken identification or an error in the literature. Thus there appears to be a long gap between American and European cyathurans, which is consistent with Burbanck's hypothesis.

The present authors agree with the generic designation, but question the specific determination of the eastern American cyathurans as *Cyathura carinata* (Krøyer) on two grounds. First, there are some disturbing discrepancies, both between descriptions and, more importantly, between specimens of *C. carinata* collected in Europe (including the type locality and vicinity), on the one hand, and our American specimens on the other. These differences will be discussed later. Secondly, on *a priori* grounds, it seems inconceivable that a species apparently limited in its habitat requirements to estuarine conditions (Burbanck, 1959a, 1959b) could become so widely dispersed without undergoing speciation as a consequence of ecological segregation and hence reproductive isolation. (Indeed, on the same theoretical basis, one might even expect some evolutionary divergence to have occurred in the presumably segregated cyathuran populations along the

entire eastern seaboard or at least at the extremes of the range, assuming sufficiently long periods of isolation.)

Pending the present systematic revision, the authors have deemed it advisable to refer to the eastern American forms as *Cyathura* sp. The genus is certainly correct, as the species fits the original brief generic diagnosis given by Norman and Stebbing (1886, p. 121) and, of course, their description of the genotype since that was based on American specimens. It also fits the longer description (quoted below) of the genus given by Barnard (1925, p. 139) in his revision of the Anthuridae.

Genus Cyathura Norman and Stebbing, 1886

"Eyes typically present, sometimes absent. Peraeon typically with dorsolateral keels and dorsal pits. Pleon with sutures indistinct dorsally. Telson lenticular in cross-section, thin, smooth. Antenna 1 with flagellum 1-3-jointed, sometimes brush-like in ♂. Antenna 2 with flagellum of a single joint. Mandible with 3rd palpal joint usually larger than 1st, with rather large apical tuft of setae. Maxilliped 4-jointed [counting a basal joint anchylosed to head]. Peraeopod 1 with more or less pronounced tooth on palm of 6th joint, unguis typically long. Peraeopods 2 and 3 with 6th joint cylindrical. Peraeopods 4–7 with 5th joint underriding 6th. Pleopod 1 not indurated. Uropods not indurated. Exopod folding over telson. Oostegites 3 pairs (in *siamensis* and also, *apud* Harger, in *"Anthura polita"* = *C. carinata*)."

Despite the fact that the type species was described from American specimens erroneously thought to be identical with it, there is no point in designating the American species as the new genotype since Norman and Stebbing's intention was clear to establish *Anthura carinata* Krøyer as the type by monotypy. The name, *"Cyathura carinata* (Kröyer)," has line precedence to the description and, in our opinion, represents the valid genotype. For its description, the original one of Krøyer (1847) and those of subsequent writers (*e.g.* Schiödte, 1875, 1876) may be consulted.

Including the type species, thirteen species of *Cyathura* have been described, but, as indicated in the following list, some have or possibly should be transferred to other genera.

Name	Distribution
Cyathura carinata (Krøyer, 1847) (type species)	Widely distributed in Europe, etc.; see text
C. crucis Barnard, 1925	West Indies
C. curassavica Stork, 1940	Netherland Antilles
C. eremophila Monod, 1925	Mauritiana, West Africa
C. estuarius Barnard, 1914 (= *C. carinata*, Barnard, 1925)[3]	South Africa
C. indica Barnard, 1925	Singapore, Siam, Paumben, Quilon, Travancore (Barnard, 1935)
C. liouvillei Monod, 1925 (= *Anthelura*?)	West Africa

[3] Comparison of paratypes (all female) of *C. estuarius*, kindly sent by K. H. Barnard, with European specimens of *C. carinata* indicates that he may have been mistaken in assigning the former to the synonomy of the latter. Examination of adult males from South Africa would be needed to determine whether or not *C. estuarius* should be resurrected.

Name	Distribution
C. milloti Chappuis, Delamare Deboutteville, and Paulian, 1956	Reunion, Madagascar
C. munda Menzies, 1951	Central California
C. pusilla Stebbing, 1904 (= *C. indica* Barnard, 1935)	Ceylon and British East Africa
C. robertiana Monod, 1925 (= *Anthelura robertiana*, Monod, 1925)	Morocco
C. siamensis Barnard, 1925	Siam
C. truncata Hansen, 1916 (= *Anthelura truncata*, Barnard, 1925; and Monod, 1925)	Davis Strait, Canada

We now propose to add a fourteenth name to the list by removing Stimpson's *Anthura polita* from the synonomy of *Cyathura carinata* (K.) and reestablishing it as a distinct species in the genus *Cyathura*. From the preceding discussion, it is apparent that Stimpson's name is not only available but appropriate in the new combination as follows.

Cyathura polita (Stimpson, 1855), New Combination

Synonomy

Anthura gracilis, De Kay, 1844, p. 44, pl. 9, fig. 34 (*non A. gracilis* Montagu).
Anthura polita Stimpson, 1855, p. 393; Harger, 1879, p. 162; Harger, 1880, pp. 398–402, pl. 11.
Anthura brunnea Harger, 1873, pp. 426, 428, 572–573.
Cyathura carinata, Norman and Stebbing, 1886 (*in partem, non Cyathura carinata* [Krøyer]); Richardson, 1900, p. 215; Richardson, 1905, pp. 64–66, figs. 47–50; Burbanck *et al.*, 1956, esp. pp. 236–237; Burbanck and Burbanck, 1958, p. 346; Burbanck, 1959a, p. 22; Burbanck, 1959b, pp. 507–511.

(Other references to *C. carinata* along the eastern coast of the United States are doubtless referable to *C. polita*.)

Description

Stimpson's (1855) original description of *Anthura polita* (= *Cyathura polita*) follows (p. 393).

"Cylindrical, smooth and shining; the seventh segment nearly as large as the sixth. Head small, inferior antennae as long as the head, somewhat larger than the superior ones and placed before them; eyes very minute, black, placed rather on the sides of the head at the anterior corners. Legs of the first pair very thick, the rest slender. Abdomen short and broad. Color pale greyish, mottled. Length, 0.9 inch; breadth, 0.13 inch. Found at the depth of two inches in sand, above half-tide.

"*Hab.* Coast of the United States, at Norfolk."

The above cited description of *Anthura polita* and Harger's (1873) description of *A. brunnea*, its first synonym, are obviously much too brief and generalized for comparative purposes. Richardson's (1905) and Norman and Stebbing's accounts of the eastern American form under the name *Cyathura carinata* (Krøyer) are

somewhat better but lack the critical details which differentiate *C. polita* from *C. carinata*. Little could be gained by comparing American material with these early acounts or with more adequate descriptions of true *carinata* given by Krøyer (1847, original description), Schiödte (1875, 1876), Barnard (1925), and Stephensen (1948).

TABLE I

Comparison of Cyathura carinata (*Krøyer*) *from Europe and* C. polita (*Stimpson*) *from eastern United States*

Characteristics	*Cyathura carinata*	*Cyathura polita*
Body Length (Average)	12.3 mm. (5 spec., Dybso Fjord) 9.5 mm. (2 ♀ ♀, Copenhagen Harbor) 10.5 mm. (1 ♀, Plymouth, England)	18.0 mm. (5 spec., Salt Springs, Fla.) 18.4 mm. (10 spec., Stony Brook, Mass.)
Head and Appendages		
General (Fig. 1)	Median rostral point bluntly pointed. Eyes small.	Median rostral point more truncate. Eyes small.
Antenna 1 (Fig. 2)	No distinctive differences between European and American. Flagellum brush-like in males of both species.	
Antenna 2 (Fig. 2)	Grooved 2d article, with small spines near distal inner edge. 1½ times longer in ♂ than ♀.	Grooved 2d article, without small thorn-like setae near inner margin. Subequal in length in ♂ and ♀.
Mandible (Fig. 3)	Four to 6 stout setae on 3d article of palp; 2–3 long setae on 2d article of palp; 18–20 serrations on flattened cutting flange.	Thirteen to 14 stout setae on 3d article of palp; 5–7 setae on 2d article of palp; 14–18 serrations on flattened cutting flange.
Maxilla 1 (Fig. 4)	Outer lamina: with 1 large and 7 small apical teeth. Collar of subterminal bristles encircles lamina.	Outer lamina: with 1 large and 6 small apical teeth. Subterminal bristles on inner and outer edges.
	Inner lamina: No essential difference between European and American species. Relatively small with single apical spine.	
Maxilla 2 (?) + *hypopharynx* (Fig. 4)	No distinctive differences between European and American species in this complex.	
Maxilliped (Fig. 4)	Three-jointed + coalesced basal piece; essentially similar in both species.	
Pereion and Pereiopods		
Pereion	Similar in both species. First segment longest, seventh shortest. Broadly V-shaped or carinate ventrally.	
Epimera	Visible along lateral margins of all segments in both species.	
Pereiopod 1 (*Gnathopod*) (Fig. 5)	Sexually dimorphic. Propodus in ♂ slender with proximal lateral margin flattened and curved outward; propodus in ♀ swollen with no outcurved proximal edge. Palm toothed in both sexes.	Not sexually dimorphic. Propodus in both sexes with palmar tooth.

TABLE I—*Continued*

Characteristics	*Cyathura carinata*	*Cyathura polita*
Pereiopod 6 (Fig. 5)	Propodus length three times its width in both sexes.	Propodus length four times its width in both sexes.
Pleon, Telson and Appendages		
Pleon (Fig. 6)	First five pleonites fused, with partial lateral suture and indentation on anterolateral side indicating first pleonite. Paired lengthwise sutures diverge anteriorly, ending in circular area with concentric rings of attachment of pleopod muscles.	Same, except anterolateral sutures and indentation less pronounced in southern specimens. Occasional specimens show additional faint lateral sutures.
	Sixth pleonite free along entire anterior and posterior border, the latter incised middorsally.	Sixth pleonite free along anterior and posterior border except where the latter fuses posteriorly with telson on each side of middorsal line.
Pleopod 2 ♂ (Fig. 7)	Terminal complex of appendix masculinum extends well beyond rounded apical edge of endopod. Hook-crowned rod originates in angle between base of lateral lobe and tip. Tip does not extend beyond end of hooked rod.	Terminal complex of appendix masculinum does not extend significantly beyond apical edge of endopod which is obtusely produced distomedially beyond lateral lobe. Hook-crowned rod originates some distance from base of lateral lobe. Distal end of appendix extends beyond end of hooked rod.
Uropods (Figs. 1 and 4)	Outer distal margin of exopod distinctly incised.	Outer distal margin of exopod not distinctly incised, almost entire.
Telson (Figs. 1 and 6)	Sides converging posteriorly toward broadly rounded apex.	Sides subparallel anteriorly but converging posteriorly into broadly rounded apex.
	Paired statocysts present near base of telson in both species.	

Much more useful were part-by-part comparisons of representative American and European specimens, supplemented by reference to the literature. Therefore, it seems best to redescribe *C. polita* largely on a comparative basis emphasizing the characters that differentiate it from *C. carinata*. In lieu of type material of either form, European specimens unmistakably identified as *C. carinata* (K.) were compared with our eastern American *C. polita*. The European material included specimens from the type locality (Copenhagen Harbor) and vicinity (Dybso Fjord, Insel Fehmarn, Ulfsund), and Plymouth, England. The American material comprised extensive collections made by Burbanck along the eastern American sea-

coast in connection with his ecological studies, and the American collections labelled "*Cyathura carinata*" in the U. S. National Museum, kindly made available to us through Dr. Fenner Chace. To facilitate the study, representative specimens of both sexes were selected from two European and two American localities for complete dissection and part-by-part comparison, but specimens from other localities were also included.

The analyses involved not only comparisons of previously described characters, but also attempt was made to find others of systematic significance. In the latter category, the appendix masculinum of the second pleopod of the mature male deserves special mention. This structure has been described in *C. carinata* and other anthurids by various authors (*e.g.* Omer Cooper, 1916; Barnard, 1925), but surprisingly little systematic use has been made of it in this group. The taxonomic importance of inherently stable genital structures is generally recognized since they presumably are not subject to environmental modification, and may, in some instances, actually determine by their structural conformations whether or not interbreeding can take place. As will be seen, the appendix masculinum serves as a valuable diagnostic character in the present study. Another objective was to determine the degree of intraspecific variation in taxonomic characters as this relates directly to their reliability.

The results of the comparison are shown in Table I and Figures 1–7. Although many of the differences indicated seem rather minor, they are consistent and collectively impressive. The major distinctions are: (1) size, (2) the many differences in detail of the mouthparts, (3) the sexual dimorphism in the gnathopods of the European species and the lack of it in the American form, (4) the difference in the articulation of the telson with the sixth pleonite in the two forms, (5) the shape of the telson, (6) the deeper incised exopod of the uropod in *Cyathura carinata*, and (7) the differences in the appendix masculinum.

As to size, *Cyathura polita* seems considerably longer than *C. carinata*, judging from available specimens. Our sample of the latter, however, is too small for a fair comparison. In the literature, *C. carinata* is reported as ranging up to 27 mm. in length. The range in length of the eight specimens of *C. carinata* that we examined was 9.3–14.2 mm., whereas the measured samples of *C. polita* ranged from 15.2 to 20.2 mm. (Salt Springs, Fla.) and from 14 to 21 mm. (Stony Brook, Mass.). The two largest specimens of *C. polita* in our collection are a female from Silver Glen Springs, Lake George, Fla., which is 23 mm. in length, and a male from Pocasset River, Mass., which is 25 mm. long. A statistical comparison of random samples of adults of the two species would be needed to determine whether there actually is a significant difference in size between the two species, but present data certainly indicate that *C. polita* is larger than *C. carinata*.

Regarding mouthparts, perhaps the most important differences are seen in the mandibles. There is much greater setation of the second and third articles of the palp in American as compared to European forms, and fewer teeth on the serrated, subapical cutting lobe in American than in European forms. The postmandibular mouthparts also exhibit differences.

The sexual dimorphism in the propodus of the gnathopod in *Cyathura carinata* has not hitherto been reported. The lack of it in American cyathurans comprises an important diagnostic characteristic.

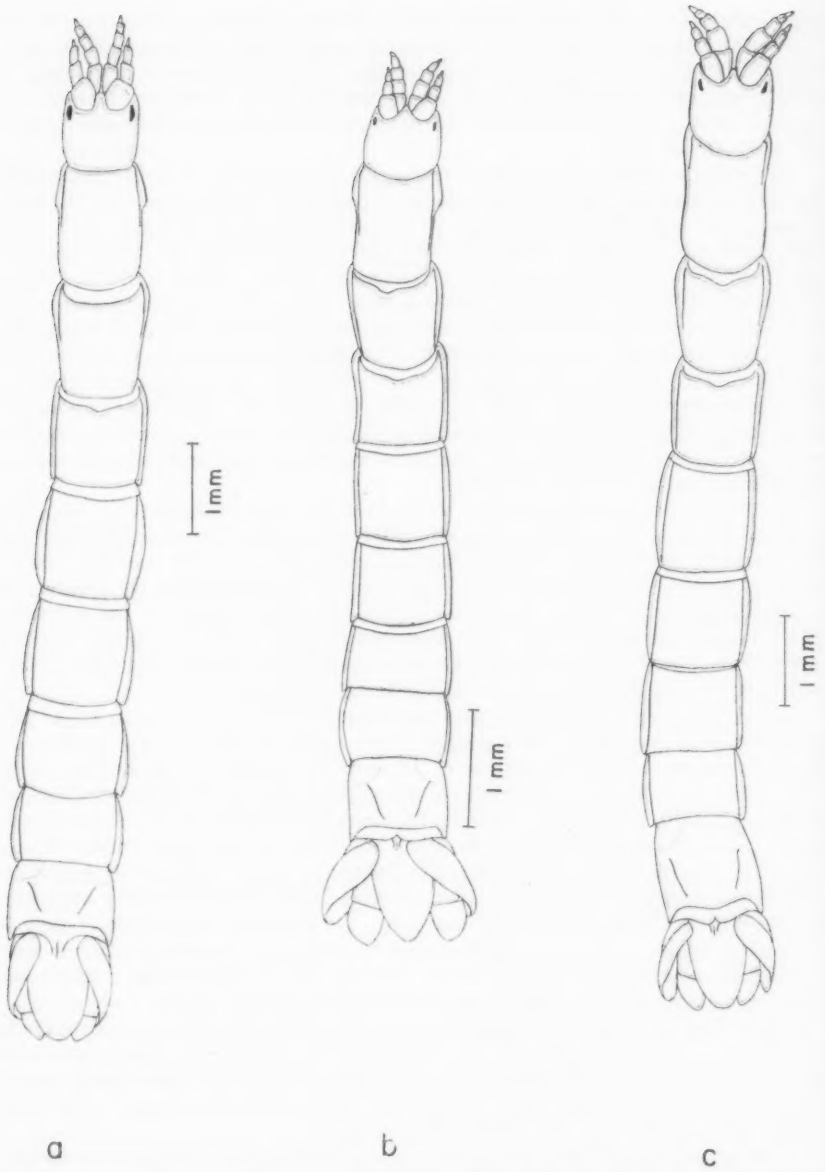


FIGURE 1. American *Cyathura polita* (Stimpson) and European *C. carinata* (Krøyer), dorsal views of females (setae, pigmentation, and pereiopods omitted for clarity) from Suwanee River, Fla. (a); Copenhagen Harbor, Denmark (b); and Plymouth, England (c).

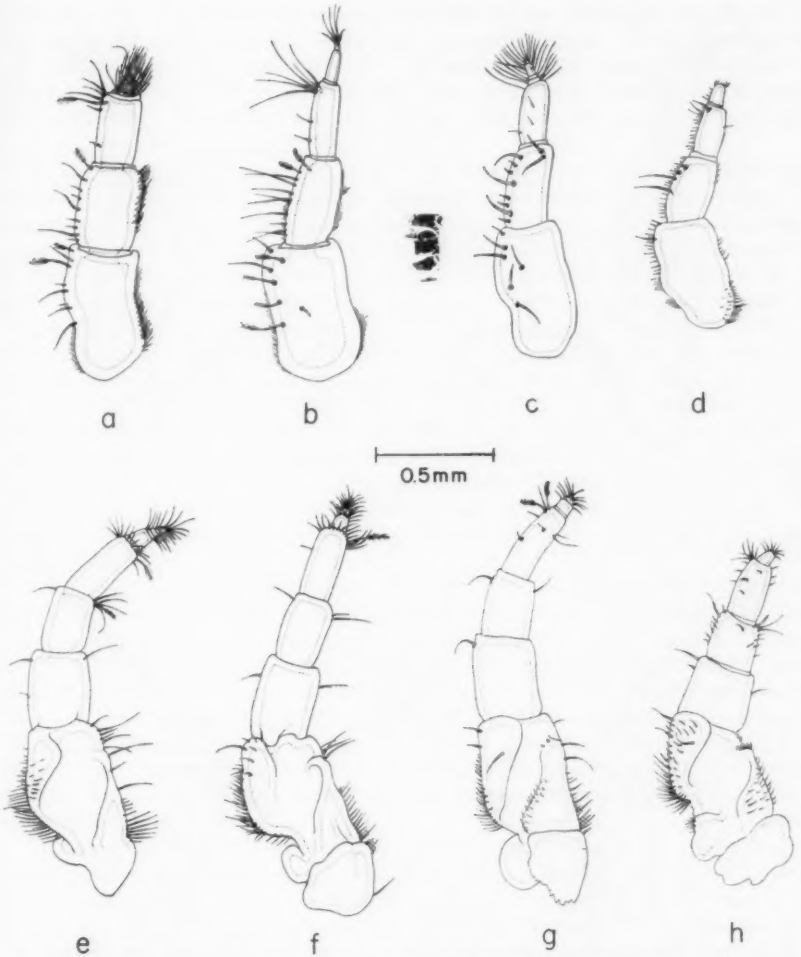


FIGURE 2. Antennae of *Cyathura polita* (Stimp.) from Suwanee River, Fla., and European *C. carinata* (K.) from Dybso Fjord, Denmark. First antennae: *C. polita*—(a) ♂, (b) ♀; *C. carinata*—(c) ♂, (d) ♀. Second antennae: *C. polita*—(e) ♂, (f) ♀; *C. carinata*—(g) ♂, (h) ♀.

The tabulated differences between *Cyathura carinata* and *C. polita* in the articulation of the telson with the pleon, in the shape of the telson, and in the apical incision of the uropodal exopod are fairly easily recognizable, given the proper view. The articulation of the telson with the free sixth pleonite may be obscured in the critical middorsal area by a heavy fringe of setae along the posterior border of the pleon, but can be seen if these are removed (Figs. 6a and 6c). The lateral

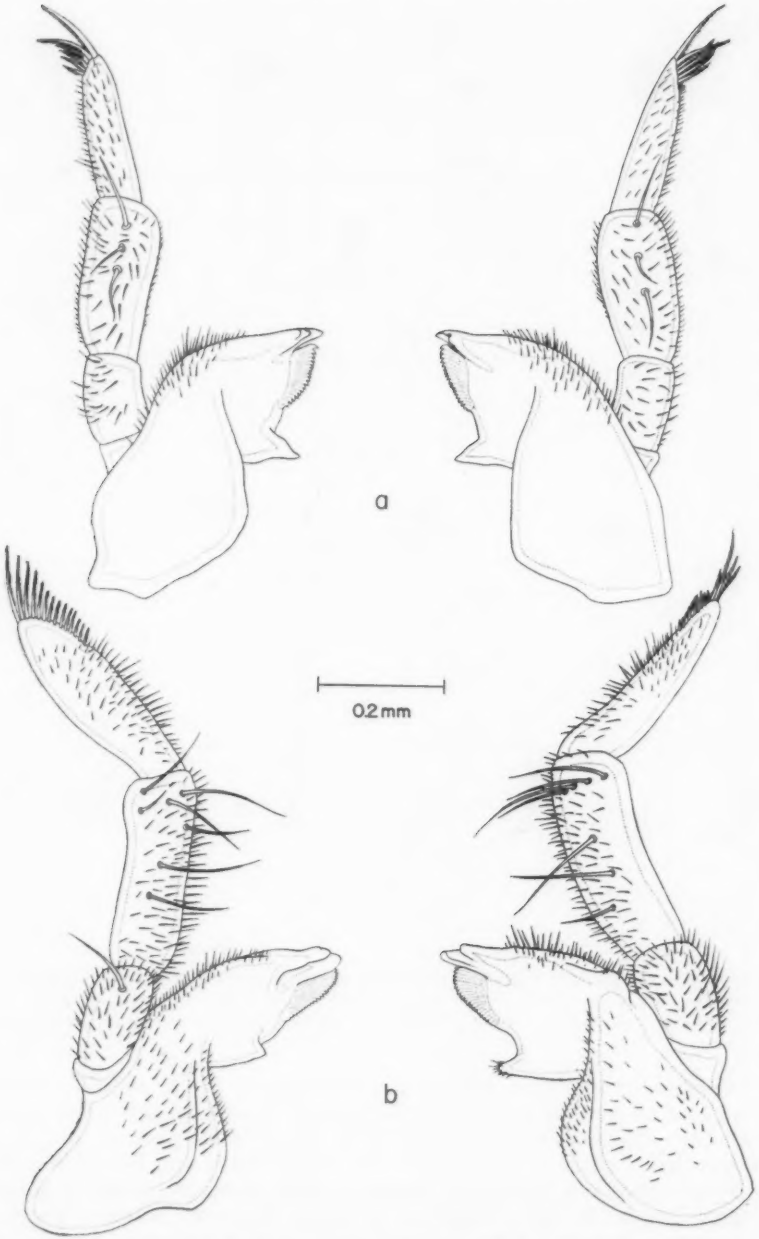


FIGURE 3. Mandibles of *Cyathura carinata* (K.) from Dybso Fjord, Denmark (a); and *C. polita* (Stimp.) from Lake George, Fla. (b).

margins of the telson may be covered anteriorly by the exopod of the uropod, but they can readily be observed if the exopods are spread apart. Then the subparallel sides of the telson in *C. polita* may be contrasted with the tapering margins of the telson in *C. carinata* (Figs. 6a and 6d). The deep apical incision of the exopod of the uropod in *C. carinata* (Figs. 6f and 6g), which is slight or absent in *C. polita* (Fig. 6e), can best be seen in lateral view.

Since the appendix masculinum has not hitherto been used extensively as a diagnostic characteristic, additional comments concerning it are in order. Attention was first called to this structure by Omer Cooper (1916) who described it from males of *Cyathura carinata* (K.) taken from brackish water in Christchurch Harbour, Hants. Subsequently, Barnard (1925) gave figures of it for several other anthurids. Presumably, these appendices serve as copulatory organs (as in isopods generally), but the details of the process and the function of their constituent parts are unknown. It develops along the inner edge of the endopod of the second pleopod of maturing males, becoming separated from it as a long, hollow, cylindrical structure, probably in the course of one or more molts. Successive stages in its separation are indicated by a series of specimens shown in Figure 7 (u, v, w). In some anthurids, it may extend far beyond the distal margin of the endopod, as in *C. milloti* Chappius, Debouttevillle and Paulian and *Pseudanthura lateralis* Richardson, in which two species it appears to be about twice the length of the endopod. It is distinctly jointed about a third to a half of its length from the base.

The apex varies among different anthurids. It is simple and club-shaped in *Cyathura munda* Menzies; slender, pointed and plumose in *Calathura brachiata* (Stimpson); slightly recurved at the tip in *Cyathura crucis* Barnard; strongly recurved like a crochet hook in *Pseudanthura lateralis* Richardson; with a lateral apophysis coiled like a ram's horn in *C. milloti* Chappius, Debouttevillle and Paulian; provided with a laterally-projecting, subterminal lobe in *Accalathura crenulata* (Richardson); and the most complex of all in *C. carinata* (Krøyer) and *C. polita* (Stimpson). In the latter two, there is a subterminal apophysis as in *A. crenulata*, but this bears in addition a hollow rod-like process capped with a crown or recurved teeth (Fig. 7, a–r).

Although the sexual stylets of European and eastern American cyathurans are constructed on the same basic plan, there are some significant differences in detail. Our observations on European *Cyathura carinata* agree with those of Omer Cooper (1916) that the appendix masculinum in that species extends well beyond the distal edge of the endopod and that the hook-crowned rod extends beyond the tip of the stylet. They do not agree, however, on the point of origin of the rod, or on the shape of the end of the subterminal lobe. According to Omer Cooper's descriptions and figures, the rod springs from the center of the flattened, subterminal lobe which is squarish at the tip. According to our observations of this structure in several males from Denmark, however, the rod originates at the base of the subterminal lobe which is bluntly rounded, rather than truncate, at the tip. The rod of the appendix in *C. polita* males, on the other hand, always originates a significant distance away from the point of origin of the subterminal lobe—anywhere from a quarter to three-quarters of the distance from the base of the lobe

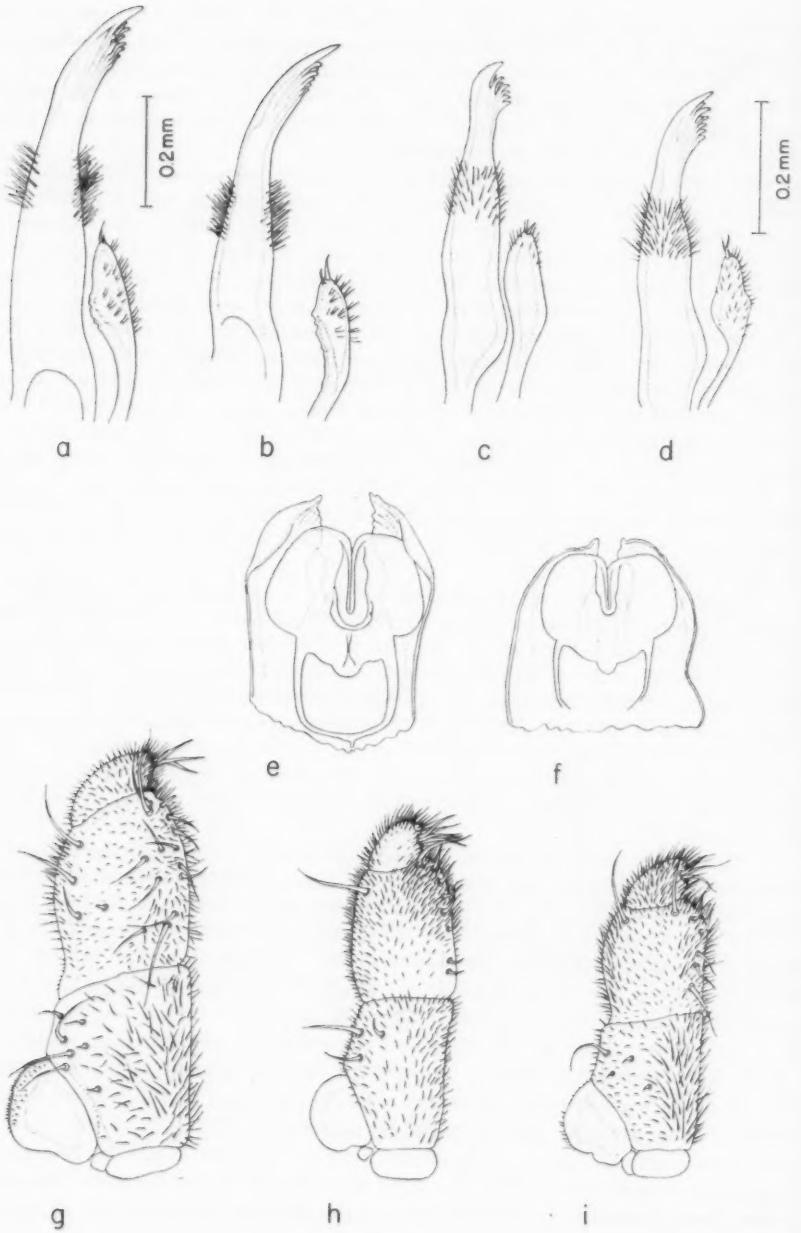


FIGURE 4.

to its tip. The authors consider the foregoing differences in the sexual stylets of *C. polita* and *C. carinata* to be of great systematic importance.

It would be of taxonomic interest to compare the chromosomes of the two species. Burbanck and Burbanck (1958) made a preliminary report of a haploid (n) number of five for *Cyathura* sp. (= *C. polita*). Subsequent study suggests that the material on which this number was based was a stage during meiosis in the male when a larger number of chromosomes became associated into five or six groups. In addition to studying meiotic and premeiotic material, division figures have been observed in smears of developing embryos and in the somatic cells surrounding the testes. Counts of 12, about 24, and about 40 have been made, and as suitable material becomes available, further work will be done to try to establish the correct chromosome number for *C. polita*. The chromosome picture of *C. carinata* is as yet entirely unknown.

Mention has been made in the literature (*e.g.* Richardson, 1905, pp. 64–66) of intraspecific variation and some deviations have been noted in the present study. One of the variations involves the degree of fusion of the anterior five pleonites (the sixth is free). Always these are fused dorsally (a generic trait) and usually also laterally. A partial suture between the first and second pleonites is generally present ventrolaterally, however, and in some specimens three additional suture lines may be faintly indicated behind the first—a complete complement of four separating the first five pleonites. In occasional specimens, there may be only a slight indentation on the ventrolateral margin between the first and second somite (Fig. 6, i–j) with perhaps a faint line extending vertically a short distance from it. In the great majority of specimens, however, the first pleonal partial suture is quite distinct (Fig. 6, e–h). These variants are just as apt to occur in New England populations as in cyathurans in Florida. Random variation and anomalies might be expected in such vestigial structures.

Some intraspecific variation was encountered in the apex of the appendix masculinum (see Fig. 7, a–r) in both European and American species, but the essential characteristics distinguishing them were always apparent. Harger (1880, Pl. 11, Fig. f) shows the second pleopod of *Anthura polita* bearing a cylindrical stylet with a simple apex lacking any indication of the characteristic subterminal apophysis with its hooked rod (see also Richardson, 1905, p. 64, Fig. 47f). It is strange that this presumably rare variant should represent the only previous portrayal in the literature of the appendix masculinum in the American species! That Harger was aware of the typical masculine appendix is presumed from the fact that two of his slides (Nos. 188 and 191, Peabody Museum, Yale University, labelled *"Anthura brunnea"*) show the second male pleopods with the complex apex of the sexual stylet characteristic of *Cyathura polita*. The specimen he dissected was taken Aug. 28, 1874, in Noank Harbor, Connecticut. The possibility has been suggested that Harger's figure represented an immature or a "first form" male, but these ideas were largely dispelled by the observation that the lateral

FIGURE 4. Postmandibular mouthparts of American and European *Cyathura* from various regions—New Jersey (a), Mass. (b, e, g), Denmark (f, h), and England (d, i). First maxillae: *C. polita* (a, b); *C. carinata* (c, d). Hypopharynx-second maxilla complex: *C. polita* (e); *C. carinata* (f). Maxillipeds: *C. polita* (g); *C. carinata* (h, i) (d and i drawn to same scale, all others same as a).

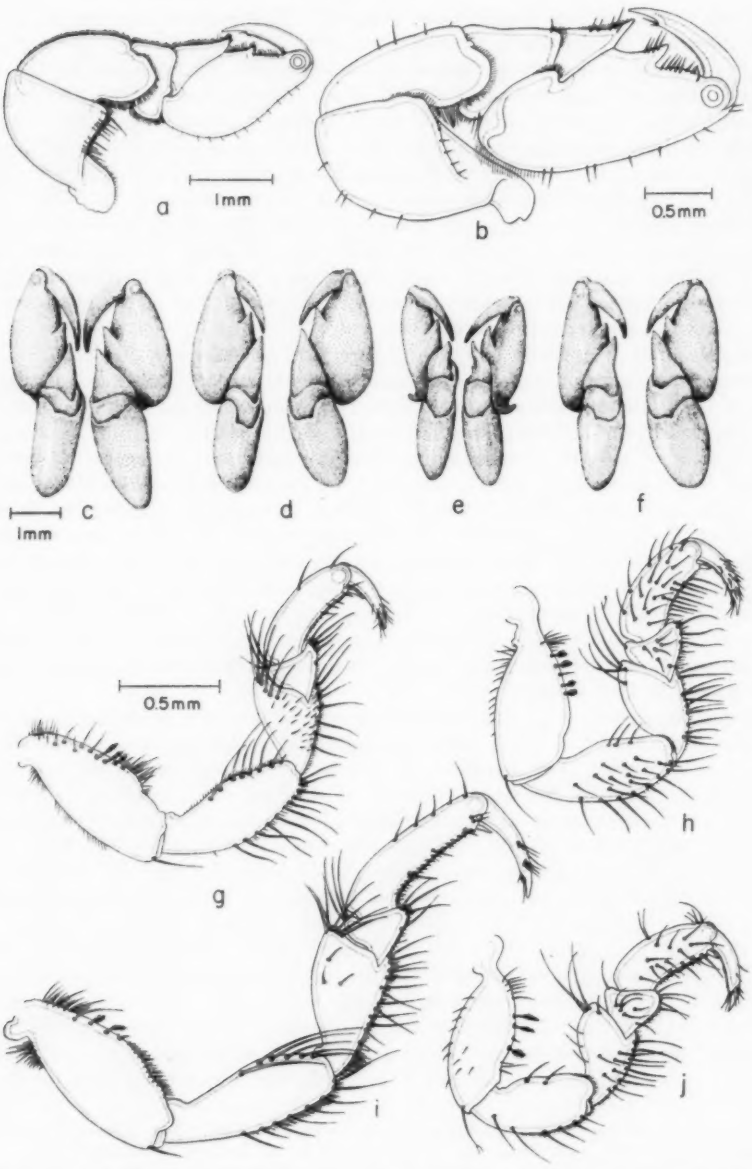


FIGURE 5. Gnathopods and sixth pereiopods of *Cyathura polita* from Stony Brook, Mass. (a), and Suwanee River, Fla. (c, d, g, i); and of *C. carinata* from Dybso Fjord, Denmark (b, e, f, h, j). Gnathopods: *C. polita*—(a)♂, (c) ♂, (d) ♀; *C. carinata*—(b) ♂, (e) ♂, (f) ♀. Pereiopod 6: *C. polita*—(g) ♀, (i) ♂; *C. carinata*—(h) ♀, (j) ♂. Note sexual dimorphism especially in propodus of gnathopods in Danish specimens, but not in American specimens.

apophysis is indicated even before the stylet separates from the endopod (Fig. 7, u, v), and by the fact that the simple apex is apparently a rare phenomenon. The authors have found only one male of the many examined that shows an appendix comparable to that illustrated by Harger. This was from a sizable male (16 mm.) in the collection from St. Louis Bay, Miss. It was the only male in the collection. Incidentally, the pleon of the Mississippi male with the anomalous appendix showed laterally faint indications of three partial sutures behind the clear-cut first pleonal partial suture. An essentially similar condition is shown in a lateral view of the pleon by Harger (1880, Pl. 11, Fig. g). We do not know whether this structure belonged to the male with the simple appendix or if there is any correlation between the two anomalies.

A study of the dorsal chromatophore pattern of *C. polita*, with emphasis on possible geographically correlated variations, is currently being made by the junior author of this paper. Preliminary observations show that the general outlines of the pattern are the same for all the specimens of *C. polita*, but that there are variations of one part of the pattern of the first thoracic segment which are characteristic of certain geographical locations.

It is significant that many of the differences, which we observed by comparing *specimens* of *Cyathura carinata* and *C. polita*, can also be seen if one compares Schiödte's (1875, Pl. 4) figures of *C. carinata* with those given by Norman and Stebbing (1886, Pl. 27) for what they thought was the same species. Schiödte's careful illustrations were doubtless drawn from European material, whereas Norman and Stebbing admittedly based theirs on American specimens of *Anthura brunnea* (= *C. polita*) which they considered to be a synonym of *C. carinata*.

The differences in detail of the mouthparts as shown in the cited figures confirm our observations on these structures. Take the mandible for example: Schiödte shows four stout setae on the terminal joint of the palp, whereas Norman and Stebbing show 13—our counts are 4–6 for *C. carinata* and 13–14 for *C. polita*. Schiödte shows 20 serrations on the subapical flange, whereas Norman and Stebbing show 15—our counts for *C. carinata* ranged from 18 to 20, compared to 14 to 18 for *C. polita*. Or take the first maxilla: Schiödte shows the outer lamina with one large and seven small apical teeth and encircled by subapical bristles, whereas the British authors show one less apical tooth and subapical bristles only on the outer and inner margins—the same differences are shown in our figures of *C. carinata* and *C. polita* (see Fig. 4, a–d).

Besides the mouthparts, comparisons of other structures shown by Schiödte and by Norman and Stebbing indicate that these authors were dealing with distinct species. One more example will suffice: The shape of the telson, as portrayed by Schiödte, with its convex, posteriorly-converging sides is quite different from that given by Norman and Stebbing who show a strap-like telson with subparallel sides—this difference is an important distinction between *C. carinata* and *C. polita* (see Fig. 6, a–d). Unfortunately, neither of these early authors drew the appendix masculinum.

Collectively, the morphological differences detailed above, together with the zoögeographical considerations previously discussed, clinch the case for specific distinctness of *Cyathura polita* (Stimpson) and *C. carinata* (Krøyer).

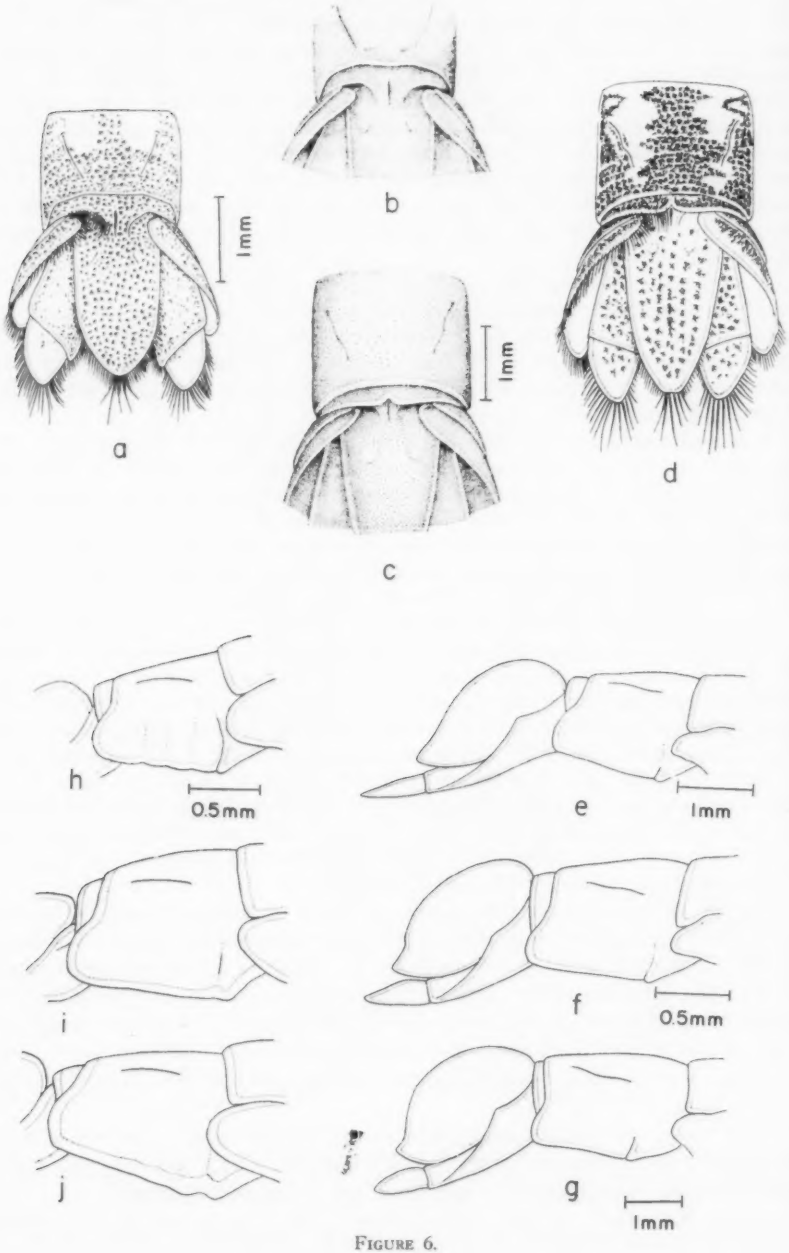


FIGURE 6.

Localities

The geographical and ecological distributions of *Cyathura polita* along the eastern coast of the United States have recently been reported by Burbanck (1959b). The list given below comprises American localities of collections labelled *Cyathura carinata* (= *C. polita*) in the United States National Museum (USNM), locations where Burbanck (WDB) and his associates have collected this species, and certain collection sites mentioned in the literature. The list shows a long coastwise distribution of *C. polita* in lakes, bays and streams from Lake Pontchartrain, La., to Chewonki Creek, Me. Every state in this range is represented, often by several localities. On the basis of critical recheck of previous determinations, three localities (specified below) should be deleted from the distributional list of U. S. National Museum specimens given by Burbanck (1959a, p. 508).

LOUISIANA: Lake Pontchartrain (USNM 97972, also WDB).
MISSISSIPPI: St. Louis Bay (WDB).
ALABAMA: Perdido Bay (WDB).
FLORIDA: Buckhorn Creek, Florida Bay near Flamingo (R. B. Manning), Lake George, Lake Poinsett (USNM 98538), Punta Rassa (USNM 25159), St. John's River (WDB), Suwanee River (WDB). *Delete:* Frankfort Bank, Key West (USNM 68401) and Friend Key Lake (USNM 44278), as the specimens from these two localities belong to a different species of *Cyathura*.
GEORGIA: Ogeechee River (USNM 98537, also WDB), Sapelo Island (A. E. Smalley), St. Mary's River (WDB).
SOUTH CAROLINA: Ashepoo River (WDB), Cooper River (USNM 86318), Edisto River (USNM 98536), Winyah Bay (USNM 42563).
NORTH CAROLINA: Calico Creek (near Beaufort) (WDB). *Delete:* Beaufort (USNM 86338 and 86339) as these anthurids have a completely segmented pleon which eliminates them from the genus *Cyathura*.
VIRGINIA: Norfolk (Type locality, Stimpson, 1855), Potomac River (USNM 81724).
MARYLAND: Chesapeake Beach (Neotype locality), Chester River (USNM 42093).
DELAWARE: Drawyer Creek (tributary Appoquinimink Creek, one mile north of Odessa) (WDB).
NEW JERSEY: Wading River (WDB).
NEW YORK: Hudson River (at Beacon, USNM 86316; at Haverstraw, USNM 86317).
CONNECTICUT: Noank Harbor (USNM 35927).
RHODE ISLAND: East Providence (USNM 19578).
MASSACHUSETTS: Buzzard's Bay, Cape Cod and the Islands (38 localities) (WDB), Danversport (USNM 41880), Gloucester (Harger, 1880), Little

FIGURE 6. Pleon and telson of *Cyathura polita* from Massachusetts (Centerville—a, b, h); Cider Hill Creek, Me. (e) and Salt Springs, Fla. (i, j); and of *C. carinata* from Plymouth, England (c, d, f) and Dybso Fjord, Denmark (g). Dorsal views (a–d) with setae completely or partially removed to show differences in articulation of sixth pleonite to telson. Lateral views (e–j) showing representative variations in degree of fusion of lateral partial sutures between first and second pleonites, especially noted in American specimens.

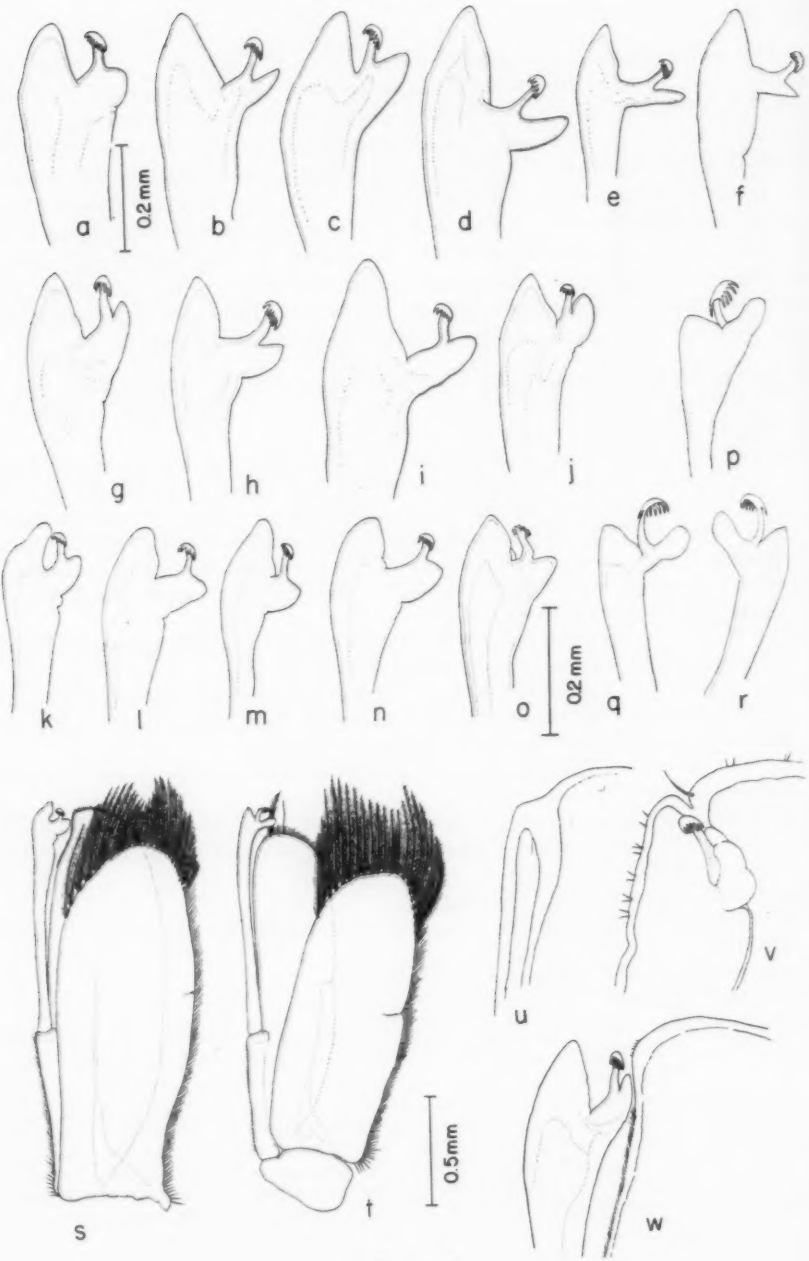


FIGURE 7.

River (near West Gloucester) (WDB), North River (near Marshfield Hills) (WDB), North Scituate (WDB), Rowley River (USNM 41881), Rocky Nook (WDB), Weir River (near Nantasket Junction) (WDB), Weweantic River (WDB).

NEW HAMPSHIRE: Exeter River (WDB).

MAINE: Chewonki Creek (tributary Sheepscot River, collected by Robert Hanks and WDB), Cider Hill Creek (tributary York River) (WDB).

Type locality and types

Although Stimpson gives the type locality of *Anthura polita* as Norfolk, Va., he does not state the disposition of the type material. Recent search for the types was made at the Philadelphia Academy of Science (a logical repository) by M. A. Miller with the assistance of Dr. F. A. Aldrich and Miss Yvonne Swabey, at the U. S. National Museum by Dr. Fenner Chace, at the Museum of Comparative Zoology (Harvard) by Dr. Elisabeth Deichmann, and at Peabody Museum (Yale) by Dr. Willard Hartman. The results were negative. Dr. Hartman, however, discovered in Peabody Museum four of Harger's slides (Nos. 188–191) of various dissected parts of his *A. brunnea* (collected Aug. 28, 1874, from mud in Noank, Conn.) and one slide (No. 286) of maxillipeds and left (?) maxilla labelled *"Anthura polita* ♀" (collected May, 1878, from mud at the shore of Squan Estuary, Gloucester, Mass.). Although the mounts were in poor condition, the parts are essentially identical with the same structures from recently collected specimens. Notably, the apex of the appendix masculinum of two males from Noank Harbor showed all the specific characteristics of *C. polita*.

In view of the practical certainty that the original types are lost, it seems desirable to establish neotypes for *Cyathura polita* (Stimpson). Accordingly, we designate a collection from Chesapeake Beach, Maryland (USNM 86340, Acc. No. 160370), as the neotype series with a male as the neotype, a female as neo-allotype, and the remaining specimens as neo-paratypes. The specimens were collected July 5, 1941, by M. P. E. Morrison 100 feet off shore, 20 feet deep from sandy bottom, and identified as *Cyathura carinata* (Kr.) by J. O. Maloney. It would be desirable, of course, to select topotypes as neotypes, but since we have no specimens from the original type locality (Norfolk, Va.) we chose a reasonably close alternate. Moreover, the neotype locality is near the middle of the known range of the species. It is unfortunate that the neotype series was taken at a rather atypical depth (20 feet), but the other reasons are overriding and dictated the choice. Harger's slide material, mentioned in the preceding paragraph, might have been designated, but the authors considered that a series of entire specimens including both sexes would serve more effectively as neotypes than dissected parts of specimens taken near one extreme of the range.

FIGURE 7. Appendix masculinum and second pleopod of males of *Cyathura polita* (a–o, s, u–w) and of *C. carinata* (p–r, t). Geographical localities: Exeter River, N. H. (a); Cape Cod, Mass. (b); Red Brook, Mass. (c); Wading River, New Jersey (d); Drawyer Creek, Delaware (e, f); Silver Glen Springs, Fla. (g, h); Salt Springs, Fla. (i); Lake George, Fla. (j, v, w); Suwanee River, Fla. (k–n, s); Lake Pontchartrain, La. (o); Cider Hill Creek, Me. (u); Dybso Fjord, Denmark (p); Insel Fehmarn, Germany (q, r, t).

Ecological remarks

The conditions of existence for *Cyathura polita* have been summarized by Burbanck (1959b) as follows (p. 509):

"1. *Cyathura* sp. live only where fresh and salt water mix.
2. The water covering the substrate where they live is never quiet for any appreciable length of time but is constantly in motion related to the slope of the land, tide, or wave action.
3. They live in simple unlined tubes of their own construction, although it is possible that they may use worm burrows as well.
4. Their substrate contains much or little sand with an admixture of vegetable debris, and at times, particularly in N. H. and Maine, blue clay."

He noted the high tolerance of this species both to wide and rapid changes in salinity in the laboratory as well as in the field, but observed that young were produced "far upstream," presumably in regions of low salinity.

He further stated, "With one possible exception, *Cyathura* sp. seem to live in waters of lower salinity in the south than they do in the north." The possible exception referred to was the Frankfort Bank, Florida, cyathurans which may live continuously in water having a salinity of over 30‰, unless springs of fresh water upwell in that region as they do in coastal waters off St. Augustine, Fla. With the finding that the Frankfort Bank specimens are not conspecific with *C. polita*, this exception to the quoted generalization is removed.

A unique location for *Cyathura polita* has recently been discovered by Dr. R. B. Manning, who found a colony burrowing in a marl spoilbank in Florida Bay at Flamingo, the southernmost tip of Florida. Since the spoilbank is situated near a canal that drains brackish water from Coot Bay and is exposed at low tide, it may be that the isopods are never exposed to the very high salinities (up to 70‰) reported in Florida Bay near these flats. It is thought that as the tide rises, the fresher, hence lighter, Coot Bay water would override the more saline water as it does in several estuaries in Massachusetts and Maine.

Recent collections of *C. polita* from the southern part of its range have revealed geographical differences in habitat not previously noted. From Maine to South Carolina, *Cyathura* can usually be found in that part of an estuary where there is an evident line of demarcation between the salt marsh grass, *Spartina*, and the less euryhaline cattail, *Typha*. The animals occur in waterways in a firm substrate composed of sand mixed with various combinations of gravel, clay, and vegetable debris. In both North and South Carolina, *C. polita* has been found by the junior author at approximately the upstream distributional limit of *Spartina*, but only where sand occurs where the muddy or otherwise soft bottom has been stabilized by the addition of shells, as at boat ramps or rock ballast at bridges. South of South Carolina and westward along the Gulf of Mexico, *Typha* and *Spartina* no longer consistently serve as indicators of the region where *Cyathura* may be found. Instead, *C. polita* is located in intertidal areas where there is a sandy substrate matted with roots and underground stems of tape grass, *Vallisneria*, and other littoral plants such as *Sagittaria*. Apparently *Cyathura* requires a relatively stabilized sandy substrate with the stabilizing factor varying with the latitude.

SUMMARY

A new combination, *Cyathura polita* (Stimpson, 1855), is established for a burrowing anthurid isopod widely distributed along the eastern and southern seaboard of the United States. Although it has long been considered conspecific with *C. carinata* (Krøyer), a predominantly European species, morphological comparisons, together with zoögeographical considerations, show the American forms are a distinct species first described by Stimpson as *Anthura polita*. Its known geographical range extends from Louisiana to Maine, with populations found in estuaries, tidal marshes, intertidal areas, etc., where salt and fresh water mix. It apparently requires a relatively stabilized sandy substrate with the stabilizing factor varying with the latitude.

LITERATURE CITED

ALLEE, W. C., 1923. Studies in marine ecology: I. The distribution of common littoral invertebrates of the Woods Hole Region. *Biol. Bull.*, **44**: 167–191.

BARNARD, K. H., 1914. Contributions to the crustacean fauna of South Africa 3. Additions to the marine Isopoda with notes on some previously incompletely known species. *Ann. So. Afr. Mus.*, **10**: 325a–442.

BARNARD, K. H., 1925. A revision of the family Anthuridae (Crustacea Isopoda), with remarks on certain morphological peculiarities. *J. Linn. Soc. London*, **36**: 109–160.

BARNARD, K. H., 1935. Report on some Amphipoda, Isopoda and Tanaidacea in the collections of the Indian Museum. *Rec. Ind. Mus.*, **37**: 279–319.

BURBANCK, W. D., 1959a. Notes on the distribution and ecology of *Cyathura* sp., an estuarine isopod. *A.S.B. Bull.*, **6**: 22.

BURBANCK, W. D., 1959b. The distribution of the estuarine isopod, *Cyathura* sp., along the eastern coast of the United States. *Ecology*, **40**: 507–511.

BURBANCK, W. D., AND M. P. BURBANCK, 1958. Chromosomes of the estuarine isopod, *Cyathura* sp. *Biol. Bull.*, **115**: 346.

BURBANCK, W. D., M. E. PIERCE AND G. C. WHITELY, JR., 1956. A study of the bottom fauna of Rand's Harbor, Massachusetts: An application of the ecotone concept. *Ecol. Monogr.*, **26**: 213–243.

CHAPPUIS, P. A., CL. DELAMARE DEBOUTTEVILLE AND R. PAULIAN, 1956. Crustacés des eaux souterraines littorales d'une resurgence d'eau douce à la Reunion. *Mem. Inst. Sci. Madagascar, sér. A*, **11**: 51–78.

DAY, J. H., N. A. H. MILLARD AND A. D. HARRISON, 1952. The ecology of South African estuaries. Part III. Knysa: A clear open estuary. *Trans. Roy. Soc. So. Africa*, **33**: 367–413.

DE KAY, J. E., 1844. Zoology of New York, or the New York fauna; comprising detailed descriptions of all animals hitherto observed within the State of New York, with brief notices of those occasionally found near its borders, and accompanied by appropriate illustrations. Vol. 1, Part 6. Crustacea. Albany: Carroll and Cook.

GOULD, A. A., 1841. Report on the Invertebrata of Massachusetts. Cambridge, Mass.: Folsom, Wells and Thurston.

GURJANOVA, E. F., 1936. *In:* Faune de l'URSS. Crustacées. Vol. 7, no. 3. Isopodes des mers orientales. *Inst. Zool. Acad. Sci. U.R.S.S., N.S. no. 6.*

HANSEN, H. J., 1916. Crustacea Malacostraca. III. The Order Isopoda. *The Danish Ingolf Expedition*, 3(5): 1–262.

HARGER, O., 1873. Isopoda. Pp. 567–573, pls. 5–6, *in:* S. I. Smith. Crustacea. Pp. 545–580, pls. 1–7, *in:* A. E. Verrill. Report upon the invertebrate animals of Vineyard Sound and the adjacent waters with an account of the physical features of the region. *U. S. Fish Comm. Rept. 1871–72*, **1873**: 295–778.

HARGER, O., 1879. Notes on New England Isopoda. *Proc. U. S. Nat. Mus.*, **2**: 157–165.

HARGER, O., 1880. Report on the marine Isopoda of New England and adjacent waters. U. S. Comm. Fish and Fisheries, pt. 6. *Report of the Commissioner for 1878*, **1880**: 297–462.

KRØYER, H., 1847. Karcinologiste Bidrag. *Naturh. Tidsskr.*, **2**: 1–123, 366–446.

KRØYER, H., 1849. Voyages en Scandinavie, en Laponie, au Spitzburg et aux Féröe. Zoologie, Crustacea. (Published under the direction of M. Paul Gaimard.) Atlas, pl. 27, fig. 3.

LARWOOD, H. J., 1940. The fishery grounds near Alexandria. XXI. Tanaidacea and Isopoda. *Fouad I Inst. Hydrobiol. Fish., Notes and Mem., No.* **35**: 1–72.

LÖWEGREN, Y., 1937. Ein für die Fauna Schwedens neuer Isopoder *Eurydice pulchra* Leach, sowie ein neues Lokal für den Isopoder *Cyathura carinata* (Kröyer). *K. Fysiogr. Sällsk Lund Förh.*, **7**: 182–185.

LUNDSTRÖM, A., 1937. Neue Fondorte für *Cyathura carinata* (Krøyer) und *Polydora ligni* Webster. *K. Fysiogr. Sällsk Lund Förh.*, **7**: 172–174.

MENZIES, R. J., 1951. New marine isopods, chiefly from northern California, with notes on related forms. *Proc. U. S. Nat. Mus.*, **101**: 105–156.

MENZIES, R. J., AND J. L. BARNARD, 1959. Marine Isopoda on coastal shelf bottoms of southern California: Systematics and ecology. *Pacific Nat.*, **1** (11): 3–35.

MONOD, T., 1925. Tanaidacés et Isopodes aquatiques de l'Afrique occidentale et septentrionale. *Bull. Soc. Sci. Nat. Maroc. Rabat*, **5**: 61–77, 233–247.

NORMAN, A. M., AND T. R. R. STEBBING, 1886. On the Isopoda of the 'Lightning,' 'Porcupine,' and 'Valorous,' expeditions. *Trans. Zool. Soc. London*, **12**: 77–141.

OMER COOPER, W., 1916. On some undescribed features in the structure of *Cyathura carinata* (Kröyer). *J. Zool. Res.*, **1**: 97–101.

RICHARDSON, H., 1900. Synopses of North-American invertebrates. VIII. The Isopoda. *Amer. Nat.*, **34**: 207–230, 295–309.

RICHARDSON, H., 1905. Monograph on the isopods of North America. *Bull. U. S. Nat. Mus.*, **54**: 1–727.

SCHIÖDTE, J. C., 1875. Krebsdyrenes Sugemund (Fortsaettelse). *Naturh. Tidsskr., ser. 3*, **10**: 211–252.

SCHIÖDTE, J. C., 1876. On the structure of the mouth in sucking Crustacea. *Ann. Mag. Nat. Hist., ser. 4*, **18**: 253–266, 295–305.

STAMMER, H. J., 1932. Die fauna des Timavo. *Zool. Jahr. Abt. Syst. Ökol. Geogr. Tier.*, **63**: 521, 656.

STEBBING, T. R. R., 1904. Gregarious Crustacea from Ceylon. *Spolia Zeylanica*, **2**: 1–29.

STEPHENSEN, K., 1948. Storkrebs IV. Ringkrebs 3. Tanglus (Marine Isopoder) og Tanaider. Copenhagen: G. E. C. Gads Forlag.

STIMPSON, W., 1855. Descriptions of some new marine Invertebrata. *Proc. Acad. Nat. Sci. Phila.*, **7**: 385–394.

STORK, H. A., 1940. A new fresh-water isopod from Curaçao. *In:* W. Hummelinck, editor. *Studies on the fauna of Curaçao, Aruba, Bonaire and the Venezuelan Islands*, **2**(10): 147–150. The Hague.

TATTERSALL, W. M., 1921. Zoological results of a tour in the far East. Mysidacea, Tanaidacea and Isopoda. *Mem. Asiatic Soc. Bengal*, **6**: 405–433.

EFFECTS OF CULTURAL VARIATION ON CELL SIZE IN A MARINE FUNGUS[1]

DON RITCHIE

Department of Botany, Barnard College, Columbia University, New York 27, N. Y.

Tests for the determination of tolerance ranges of fungi isolated from salt water have indicated that some varieties show, in laboratory culture, a definite reaction to salinity and temperature, and especially to a combination of the two factors. These organisms will grow in media containing salts in about the same concentration as that found in the sea, and at temperatures they might meet in nature. When incubated at sub-optimal temperatures, they grow faster in less concentrated salts, or at supra-optimal temperatures they grow faster in more concentrated salt media.

Interdependence of salinity and temperature has been noted in a number of organisms, with a variety of criteria being used by different investigators. Johnson (1960) used infectivity of a fungus on a barnacle. Todd and Dehnel (1960) used high-temperature resistance in crabs, as McLeese (1956) did in the lobster. In all these studies, a high salinity coupled with high temperature proved favorable. An exception is the work of Broekema (1941), who, using survival time as a criterion, found that shrimp endured a low temperature better if the salinity was high.

Conclusions on the salinity-temperature relations of fungi were based, in our work, on growth rates, with growth measured as increase in colony diameter (Ritchie, 1957). We considered growth in hyphal length to be proportional to total growth of the colony, but a question arises as to whether variations in total growth of these lower forms are reflected in the sizes of individual cells. Consequently, a series of experiments, set up so as to allow for variation of both temperature and salinity, was conducted to give information on how these factors affect the cell size of a sensitive organism.

Materials and Methods

The fungus used in the present experiments was a variety of *Phoma herbarum* West., an imperfect form isolated from yellow pine panels which had been submerged in Limon Bay, Panama. It was cultivated on agar media containing 0.5% glucose, 0.1% Difco yeast extract, and sea water which was either diluted with distilled water or concentrated by evaporation to produce a graded series containing 8, 15, 23, 30, 60, and 90 parts per thousand of salts. Cultures were prepared in triplicate, and the series at all salinities was incubated at the following temperatures: 7° C., 16° C., 25° C., 30° C., and 37° C. Figure 1 shows the results of a preliminary experiment, which indicated that at an incubation temperature of 16° C., the fungus would grow fastest in a salt concentration of about

[1] Aided by Grant G-2011 from the National Science Foundation.

20 S ‰. At 25° C., the "optimum" was about 25 S ‰; at 30° C. about 38 S ‰; and at 37° C. about 48 S ‰.

Using these data as a guide, we ran two sets of cultures, one with the temperature varied as before, but with the media made up to the salt concentration which seemed optimal for each temperature, and a second set, again with the same temperatures, but with all the media made to contain 25 parts per thousand of salts. The latter concentration was chosen because, being one which will support some growth at any temperature, it would permit comparison of various temperatures with uniform salinity.

After five days of incubation, samples were cut from the colonies just back of the advancing edge of the mycelium, where the hyphae were no longer growing.

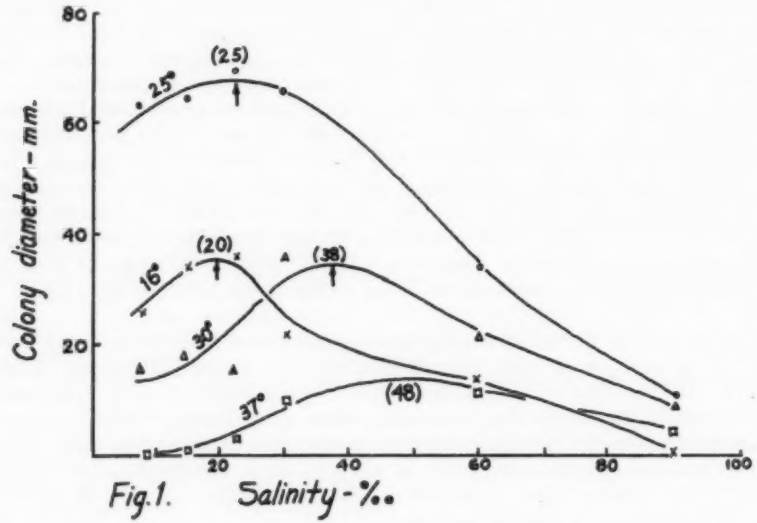


FIGURE 1. Effect of temperature and salinity on total growth of *Phoma herbarum*. The numbers in parentheses at the peak of each curve indicate the approximate salinity optimum for each temperature.

Hyphal diameters were measured with an ocular micrometer in conjunction with an oil immersion objective. Measurements were estimated to the nearest half unit of the micrometer. In any field of view, randomly picked, hyphae were measured as they fell across the micrometer scale, with subjective choice minimized as much as possible. In the experiment with salinity at 25 S ‰, fifty measurements were made on each culture; in the second, when salinity was varied, one hundred measurements were made. In one instance (25° C. and 25 S ‰), three independent, fifty-count measurements were made in order to find whether important sampling errors would show up. Inasmuch as the three runs gave consistently similar data, the number fifty was considered adequate for a sample.

Diameters only were used, and cell lengths were ignored because they are extremely variable and irregular.

The figures from any one run, as for example from 30° C. and 38 S ‰, were arranged in order, and the number of identical hyphae grouped together and counted. The resulting number was expressed as a per cent of the total count for that run, and the frequency was plotted as the ordinate, with hyphal diameters on the abscissa. Curves could thus be made comparing temperatures or salinities separately or simultaneously.

Results and Conclusions

Of the temperatures tried, the best growth of this isolant of *Phoma* occurred at 25° C. Growth at higher temperature (30° C.) or lower (16° C.) was markedly

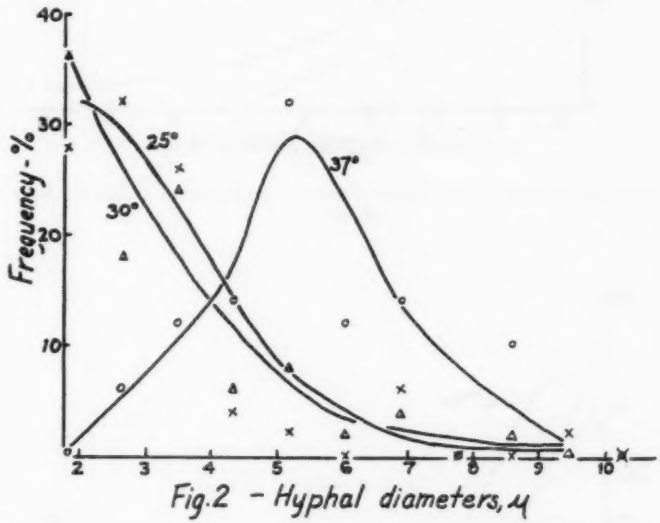


Figure 2. Effect of temperature on hyphal diameter. Percentage distribution of hyphal diameters in microns. Temperatures as indicated; all media containing 25 S ‰.

less than at the optimum, while at 37° C. it was severely retarded (Fig. 1). When cell size is considered, diameters of hyphae were, within limits, little affected by changes in temperature. Measurements from cultures incubated at 25° C. and 30° C. yielded curves that are similar (Fig. 2). Actually, the curve from the 16° C. culture is about the same, but was omitted from the graph in the interest of visual simplicity. But when the temperature was maintained at the rather high level of 37° C. with salinity kept low, the shape of the curve was altered, with a much larger percentage of thicker hyphae and a reduction in the number of slender ones. In fact, only when a definitely supra-optimal temperature was applied did the curve approach what would be expected of a normal size distribution, statistically speaking.

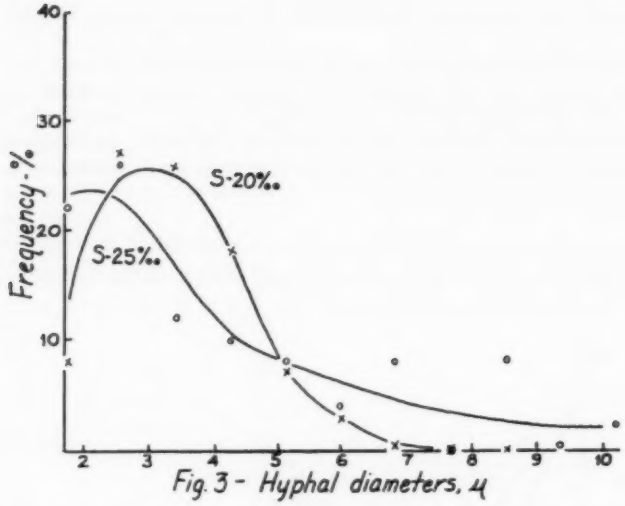


FIGURE 3. Effect of salinity on hyphal diameter. Salinities as indicated; both cultures at 16° C.

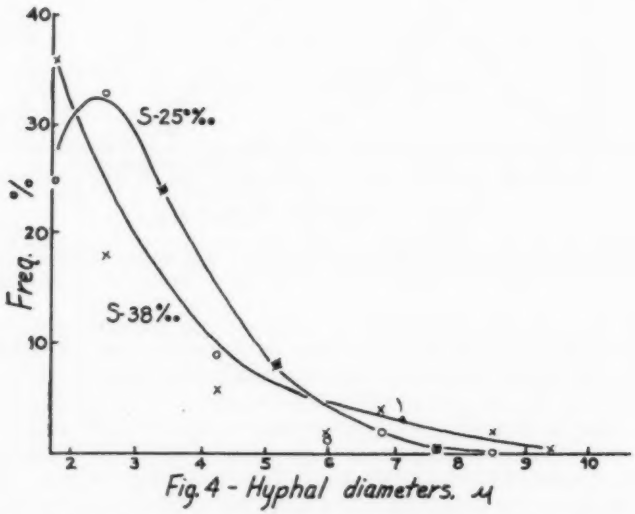


FIGURE 4. Effect of salinity on hyphal diameter. Salinities as indicated; both cultures at 30° C.

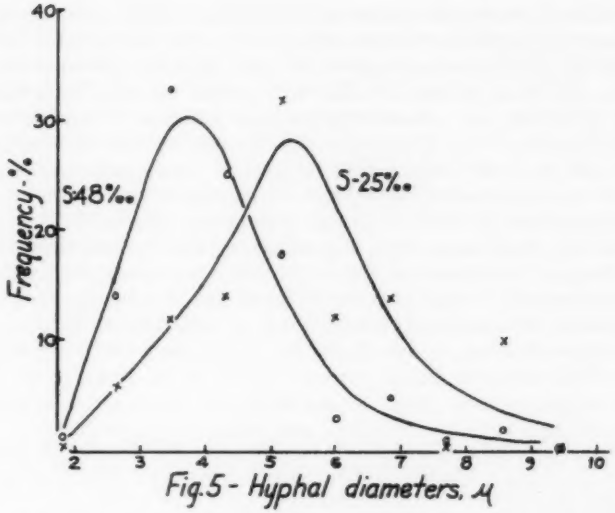


FIGURE 5. Effect of salinity on hyphal diameter. Salinities as indicated; both cultures at 37° C.

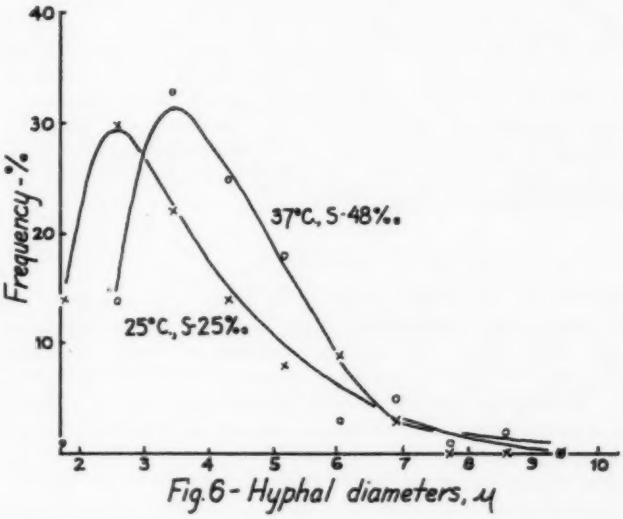


FIGURE 6. Combined effects of salinity and temperature on hyphal diameters. See text for explanation and discussion.

When salinity alone was considered, temperature being held constant, cell diameters were little altered as long as the lower temperatures were being investigated. Thus at 16° C., cultures grown at their apparent optimum for that temperature, *i.e.*, 20 S ‰, yielded data similar to those obtained from cultures from media at 25 S ‰ (Fig. 3). Essentially the same thing can be said for the cultures kept at 30° C. (Fig. 4). (Cultures at 25° C. are not treated here because the optimum at 25° C. is 25 S ‰, which happens to be the one salinity which was used for all the experimental cultures.) When, however, a run was made at 37° C., with one culture at 25 S ‰ and one at the optimal 48 S ‰, the curves show that at this high temperature the salinity of the medium made a difference in the distribution of hyphal diameters, with the lower concentration producing a more nearly "normal" curve, statistically considered, than the optimal concentration. In general, the frequency curves were strongly skewed in the direction of thicker hyphae, with relatively few of the thicker and very few of the thickest ones. Hoffman (1953) obtained similar curves, skewed to the right, in his analysis of cell size of mouse tumors. Such a distribution is not unusual in a sample like this, in which larger cells can exist, but cells below a certain minimum apparently cannot. The cultures in the higher concentration, 48 S ‰, had more narrow hyphae and fewer thick ones, in this respect resembling cultures grown at lower temperatures (Fig. 5).

This last observation is emphasized by comparison of simultaneous alteration of both temperature and salinity. When this is done, one notes a great reduction in the differences that were apparent when either factor alone was raised to a rather high level. Two curves, one from a culture from 25° C. and 25 S ‰ and one from 37° C. and 48 S ‰, are compared in Figure 6. Except for a slight preponderance of thicker hyphae in the culture from the higher temperature, the two curves are very much alike. By and large, simply raising the temperature has little effect upon the cell size of this fungus unless the temperature approaches the maximum possible, at which point cell diameters are generally increased. Similarly, raising the salinity is relatively ineffective unless the divergence in salinity is provided at a high temperature. The effect of either factor can be reduced by concomitant alteration of the other factor.

Discussion

A comparison can be made between two kinds of response of the fungus to temperature and salinity variations. One is expressed as regulation of radial growth of the entire colony, and one as regulation of hyphal diameters. A similarity between the two kinds of response appears in a review of the total temperature range. As long as the colonies are incubated at low or optimal or slightly supra-optimal temperatures, the change in salinity optima with rising temperature is relatively slight, but as the temperature approaches the maximum for the organism, the salinity optimum, broad as it is, becomes much higher. Similarly, hyphal diameters are little affected by a rise in incubation temperature through the lower registers, but as the maximum is approached, the diameters are markedly greater. It should be recalled, however, that if both factors are made to approach their maxima simultaneously, cell size scarcely changes.

The Coelenterate, *Cordylophora*, affords a comparable example among animals. Kinne (1958) recorded the sizes of hydranths and the number and size of tentacles in that organism, and found that when the temperature was low (10° C.), growth was greater in fresh than in saline water (30 S ‰), but at 20° C., the salinity relation reversed. This pattern in principle resembles that operating in *Phoma*, but the temperature effects upon ectodermal cell size of the animal were unlike those on the cells of the plant. Animals raised in fresh water showed a temperature effect in which the ten-degree animals had relatively wider cells than the twenty-degree ones; but animals raised in salt water (30 S ‰) showed an opposite reaction. In neither the animal nor the fungus, however, did cell size have a direct relation to total colony size, and the conclusion is evident that total size of the organism is very slightly or not at all dependent upon cell size, and that the mechanism by which temperature and salinity affect total growth is different from the mechanism by which those factors affect the size of individual cells.

Summary

1. When a marine isolant of *Phoma herbarum* West. was grown in a variety of temperatures and on media containing various amounts of salts in the proportions of sea water, the size of the cells, expressed as hyphal diameters, was not greatly changed unless a temperature high enough to be inhibitory (37° C.) was applied in conjunction with a relatively low salinity (25 S ‰), under which conditions more thick hyphae and fewer thin ones grew.

2. Temperature effects upon total growth, expressed as colony diameter, and upon cell size, expressed as hyphal diameters, were similar only in that a temperature approaching the maximum is required to make any considerable change in the salinity optima or in the frequency of larger cells.

LITERATURE CITED

Broekema, M. M., 1941. Seasonal movements and the osmotic behaviour of the shrimp, *Crangon crangon* L. *Arch. Néerl. Zool.*, **6**: 1–100.

Hoffman, J. G., 1953. The Size and Growth of Tissue Cells. C. C. Thomas, Springfield, Ill.

Johnson, T. W., Jr., 1960. Infection potential and growth of *Lagenidium chthamalophilum*. *Amer. J. Bot.*, **47**: 383–385.

Kinne, O., 1958. Über die Reaktion erbgleichen Coelenteratengewebes auf verschiedene Salzgehalts- und Temperaturbedingungen. *Zool. Jahrb.*, **67**: 407–486.

McLeese, D. W., 1956. Effects of temperature, salinity, and oxygen on the survival of the American lobster. *J. Fish. Res. Bd. Can.*, **13**: 247–272.

Ritchie, D., 1957. Salinity optima for marine fungi affected by temperature. *Amer. J. Bot.*, **44**: 870–874.

Todd, Mary-Elizabeth, and P. A. Dehnel, 1960. Effect of temperature and salinity on heat tolerance in two grapsoid crabs, *Hemigrapsus nudus* and *Hemigrapsus oregonensis*. *Biol. Bull.*, **118**: 150–172.

METAMORPHOSIS OF THE VELIGER LARVAE OF NASSARIUS OBSOLETUS (GASTROPODA) IN RESPONSE TO BOTTOM SEDIMENT [1]

RUDOLF S. SCHELTEMA [2]

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Characteristic of most marine organisms is propagation of their kind by the production of great numbers of pelagic larvae. As a consequence, the dispersion of such organisms over large areas is possible. Success of benthic organisms is dependent, however, upon the metamorphosis of their larvae onto a substratum which meets their special adaptations and requirements. The metamorphosis of marine larvae upon a favorable substratum, if left to chance alone, would be indeed remote.

This hazard is apparently not as great as was once supposed, since many marine invertebrate organisms delay metamorphosis until they encounter a favorable substratum. Metamorphosis occurs as the response to a stimulus received from the desirable environment. Inasmuch as the evidence has been reviewed in a recent paper by Wilson (1952), it is necessary to relate only briefly the facts which lead to this conclusion.

The earliest laboratory observations which indicated that the substratum played a role in stimulating metamorphosis were those of Mortensen (1921) on the echinoid *Mellita sexiesperforata*. Mortensen made the casual observation that when larvae which were ready to metamorphose were placed in a culture jar with sand, metamorphosis ensued within a few days, while similar larvae from the same original culture placed in a jar without sand showed "hardly any further advance in the process of metamorphosis." This observation was later confirmed on three other species (Mortensen, 1938).

Harrington (1921) observed that shipworm larvae (*Teredo norvegica*) were positively chemotactic to a substance which he extracted from wood and which he believed was malic acid. Wilson (1952) has extended this observation and has shown that in the absence of the wood, shipworm larvae do not metamorphose. Settlement may be postponed for about one week.

Wilson (1932, 1937, 1948) and Day and Wilson (1934) observed the response of larvae of several polychaetous worms to the presence of their natural substratum. In all cases the settlement of the larvae was brought on by the addition of small quantities of substratum collected from their normal habitat. Substrata from other areas either were less effective or appeared to inhibit metamorphosis. Wilson chose *Ophelia bicornis* for a more detailed study and the results of this work have been reported in a series of papers (Wilson, 1948, 1951, 1952, 1953a, 1953b, 1954,

[1] Contribution No. 1132, Woods Hole Oceanographic Institution.

[2] This paper is based on part of a dissertation presented to the University of North Carolina, Chapel Hill, N. C., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1955). While size and shape of grains seemed to have some bearing on the metamorphosis-inducing quality of the substratum, Wilson (1955) finally concluded that (p. 537) "the factor most active in inducing metamorphosis and settlement of *Ophelia bicornis* larvae is the presence on sand grains of living organisms, such as bacteria, and that these should be neither too few nor too abundant. . . . Certain species are more effective in promoting settlement than are others. . . . "

Two other species of polychaetes have shown a settlement response to the presence of substratum. Nyholm (1950) found that *Melinna cristata* (p. 88) "tended to metamorphose only in the presence of a suitable substratum rich in detritus and never when merely supplied with clear water." Smidt (1951) observed that (p. 60) "the lack of substratum will prolong larval life [of *Pygospio elegans*] while settling is furthered by the presence of a substratum." Moreover, "sterile sand and naturally occurring pebbles have an impeding effect" on metamorphosis.

In a careful study, settling of the actinotroch larvae of *Actinotrocha branchiata* [= *Phoronis mülleri*] is described by Silén (1954, p. 234): "The metamorphosis is easily and infallibly brought about by introducing some of the clay-mixed sand in which the adults are found, to a bowl containing a mature specimen." The metamorphosis which occurs "within a few usually about ten minutes" is described in great detail and involves the testing of the substratum by the larvae.

The study of Jägersten (1940) on the metamorphosis of the archiannelid *Protodrilus rubropharyngeus* is especially interesting because it describes the characteristics of a metamorphosis-inducing material. In nature *P. rubropharyngeus* has positively phototactic mature swimming larvae which, on reversing their photic response, sink to the bottom and metamorphose in a limited littoral zone with a shell or shell and pebble bottom. In the laboratory swimming larvae usually do not metamorphose but may live for two weeks without signs of transformation. If a shell or pebble from the normal habitat is included in the culture vessel, the larvae metamorphose within twenty-four hours. The material which induces this metamorphosis may be transferred to the water. It is resistant to heat, acids, and alkalis and is apparently associated with suspended material which may be removed either by centrifugation or filtration. Jägersten concluded that some non-living inorganic material is responsible for the settling response.

Wilson (1952) has concluded (p. 53 ff.): "It is evident that larvae do not settle just anywhere and that many of them have some power of selection. Over what period of time this power can be exercised is not always so clear; neither is the mechanism whereby larvae are enabled to make the selection, whether by touch, smell or other means. . . . It has proved easier to establish the facts that metamorphosis can be delayed and that larvae can choose their substratum . . . than to determine the actual features of the environment, physical, chemical or biological, perceived by the larvae and to which they react."

One of the more conspicuous organisms on intertidal flats along the Atlantic coast from the Gulf of St. Lawrence to northern Florida is *Nassarius obsoletus* Say. This prosobranch gastropod is extremely abundant in its preferred habitat. Newly metamorphosed snails, during favorable years, occur in large aggregations which reach densities of 23,000 individuals per square meter. A patch of newly metamorphosed snails, while usually not uniform in density, may exceed 100 square

meters in area. Apparently success of the species is due to the settlement of the larvae in a favorable environment; an explanation may be that the larvae metamorphose in response to some clue provided by the environment.

The experiments which are described in the following pages are offered in support of such a hypothesis. They show that in the laboratory, the larvae of the gastropod mollusk *N. obsoletus* are able to recognize a favorable substratum and can, to a considerable degree, delay metamorphosis until a suitable substratum is come upon. Hence the "power of selection" in choosing a place for settlement is proven to exist in yet another group of invertebrate animals. The experiments also suggest the singular manner by which the larvae achieve this end.

I should like to acknowledge with gratitude the financial assistance afforded through a fellowship from the Woods Hole Oceanographic Institution during the summers of 1956, 1957, and 1958, and the encouragement offered by Dr. Bostwick H. Ketchum of that institution.

Mr. Harry J. Turner has been most generous in making available all the facilities of his laboratory. At various times during the course of the research he offered comments and suggestions which were helpful and for which I am indebted.

I am grateful to Dr. Charles E. Jenner for his suggestions and advice at certain critical points of the research and for his careful reading of the manuscript.

With Amelie, my wife, I have shared some exciting moments of discovery, but also many evenings counting over larvae and editing the manuscript.

Experimental Procedures

The experiments that have demonstrated the relationship between the substratum and metamorphosis in *Nassarius obsoletus* have been simple in their design. Inasmuch as all of these experiments involved quite similar methods, the general procedure used will be described here; the details are presented later together with the results.

All experiments entailed the use of at least one control and one experimental container, each with twenty veliger larvae. The containers, which were of translucent polyethylene,[3] were filled with one-half liter of sea water that had been filtered through glass or dacron wool.

The bottom sediment to be tested was placed in the experimental dishes. About 5 cc. of substrate material were required to just cover the bottom of a container. The substratum, unless otherwise indicated, was screened before use by passing it through either a Number 60 (240 μ) or 100 (140 μ) stainless steel screen, or a Number 2 (316 μ) bolting cloth. This removed all organisms larger than *ca.* 0.3 mm, which interfered with the conduct of the experiment. The substratum usually was composed of a very fine sand and included a considerable amount of organic material.

The controls consisted of containers with filtered sea water only; in some cases, when the substratum was treated in a manner to be explained later, additional controls with untreated substratum were added to the series.

[3] Manufactured by the Kordite Corporation, Macedon, N. Y. Polyethylene to which coloring matter has been added is unsuitable, presumably because of the toxic effect of the dye.

Control and experimental containers were started simultaneously with larvae grown in and picked at random from the same culture. The larvae at the beginning of the experiment were at a stage of development which precedes metamorphosis, *viz.*, the creeping-swimming stage, attained about 3 weeks after emergence from the egg capsule. Subsequently both experimental and control containers were placed together on a sea table or nearby shelf. Hence the larvae are presumed to have been maintained under similar but not necessarily constant or uniform conditions throughout the course of their growth previous to the experiments and also, except for the conditions being tested, throughout the duration of the experiments. Temperature throughout the experiments ranged from 22° to 24° C.

Most experiments were terminated after 24 hours; some ran only 12 hours and others lasted up to four days.The larvae or recently metamorphosed young were removed from the plastic containers either by pipetting them directly from the containers or by carefully screening the contents of the containers so as to permit the substratum to pass through, leaving the organisms behind. The number of larvae that had metamorphosed was then determined by inspecting each larva under a dissecting miscroscope. The criterion used to determine metamorphosis was the loss of the velum.

A concise description of the method used in rearing the larvae will suffice, inasmuch as a more complete account will be given elsewhere, together with the external morphology of the larvae. Egg capsules were obtained in the field or from snails held in the laboratory. After emergence from the egg capsules the larvae were collected on a fine mesh screen, placed into 15-liter rearing vessels, and aerated. Water in the rearing vessels was changed every second or third day by passing the entire contents of the vessel through a fine mesh screen which retained the larvae. *Phaeodactylum tricornutum* Bohlin (Ehr.) W. Sm. at a concentration of 2×10^5 cells/ml. was used as a source of food. This diatom may be readily grown in mass culture.

A brief explanation of the experimental data given in the tables is required. Since the age of the larvae, as well as previous cultural conditions, was of necessity not identical from one group of experiments to the next, any set of experiments performed at one time are not strictly identical to those of another. The past history of the culture and the difference in the age of the creeping-swimming larvae explain much of the variability observed in the data. It is clearly valid, however, to compare the results from a series of experimental containers with those derived from their respective controls. The statistical significance of the difference between such means has been determined by the Student t-test. The percentages metamorphosed were corrected for mortality, and all statistical computations are based on the number of living organisms recovered at the end of the experiments.

Reaction of Nassarius obsoletus to the Substratum

a. *Metamorphosis in the presence of natural substratum*

The importance of the substratum to onset of metamorphosis in *Nassarius obsoletus* is readily demonstrated. In the presence of a bottom sediment on which

TABLE I

Percentage metamorphosis of Nassarius obsoletus *in the presence of of natural substratum (results from ninety-four experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed*
Natural substratum	2.1	70.0 ± 2.4
Without substratum	1.4	18.9 ± 1.4

* One standard error is indicated on all values throughout this paper.

juvenile *N. obsoletus* are found in nature, the percentage metamorphosis is very significantly greater than in controls without such natural substratum. The results of 94 experiments show that with a natural substratum 70.0% of the larvae ended their planktonic existence, whereas in controls of filtered sea water only, 18.9% of the larvae metamorphosed. The value of P in these experiments equals less than 0.001. Moreover, in not one of the above experiments did the percentage of metamorphosis in the control ever exceed that in the experimental culture. The results of the experiments are summarized in Table I. The larvae at the end of

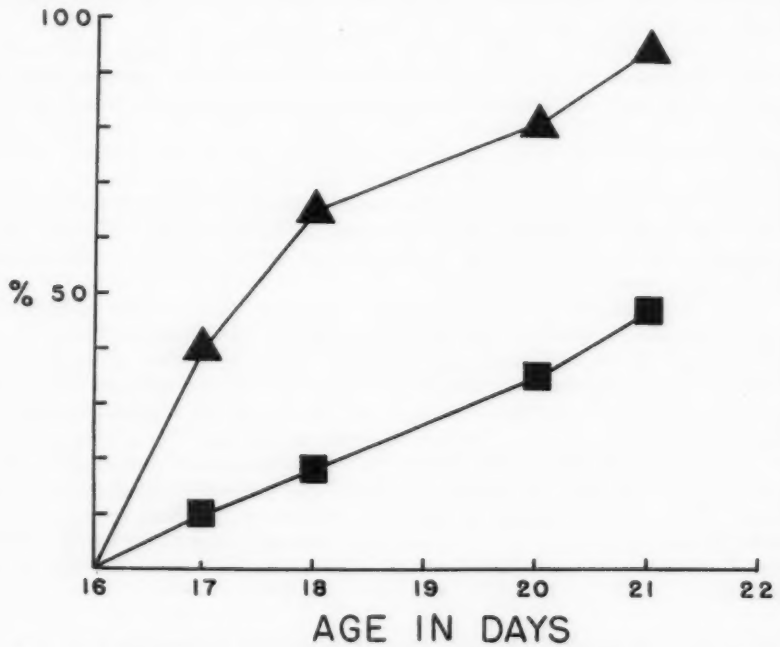


FIGURE 1. Percentage metamorphosis with (▲) and without (■) natural substratum in *Nassarius obsoletus*. Fifty larvae, 16 days after emergence from egg capsules, were placed in each of two 2-liter containers. In the bottom of one was placed freshly collected substratum; no substratum was placed in the control. The percentage metamorphosis in each container is shown at the times indicated on the abscissa.

the experiments ranged in age from 16 to 35 days (mean 25.7) after emergence from the capsule. The eggs were obtained from snail populations in the vicinities of Woods Hole, Massachusetts, and Beaufort, North Carolina.

Apparently metamorphosis cannot be indefinitely delayed. In the absence of bottom sediment the tendency to metamorphose is a simple linear function of age (Fig. 1). The maximum delay which was demonstrated was 20 days after the creeping-swimming stage had been attained. In the presence of bottom sediment the tendency to metamorphose is also a function of age; however, this tendency increases very rapidly and is not a simple linear function. In a series of experiments (Scheltema, 1956), it was shown that when 20-day-old larvae were used, 17% metamorphosed in response to bottom sediment, whereas in similar experiments using 31-day-old larvae 90% metamorphosis resulted.

TABLE II

Mechanical analyses of substrata from three areas used in metamorphosis experiments*

Mesh no.	Openings in mm.	Per cent passing through sieve		
		Substratum #1	Substratum #2	Substratum #3
5	4.000	82.8	—	—
10	2.000	75.4	—	—
18	1.000	57.5	100.0	100.0
35	0.500	24.3	99.6	97.7
60	0.250	6.3	94.4	92.0
120	0.125	0.5	66.2	84.1
230	0.062	—	7.3	77.3
Median diameter		0.9 mm.	0.1 mm.	<0.06 mm.

* Substratum #1: Head of Great Pond (off mouth of Perch Pond), East Falmouth, Cape Cod, Massachusetts (see Hulburt, 1956). "Favorable" substratum in preference experiments.

Substratum #2: Experimental area of Woods Hole Oceanographic Institution, Barnstable Harbor, Cape Cod, Massachusetts (see Ayers, 1959).

Substratum #3: Mouth of Great Pond (west shore), East Falmouth, Cape Cod, Massachusetts (see Hulburt, 1956). "Unfavorable" substratum in preference experiments.

b. *Preference for a favorable substratum*

Certain natural substrata are apparently preferred over others, inasmuch as a significant difference in the response of *Nassarius obsoletus* larvae to different substrata may be demonstrated experimentally. A sustratum sample obtained from a location shown to be very favorable for metamorphosis of *N. obsoletus* (#1, Table II), as indicated by the large number of snails observed to have metamorphosed there in the previous two years, was compared with another sample collected from a region believed to be unfavorable for metamorphosis (#3, Table II), as demonstrated by the complete absence of recently metamorphosed snails during the same two-year period. The latter region, although unfavorable for metamorphosis, was not inimical to the existence of the species, since older snails of three years or over regularly were found to inhabit this flat. Plankton tows

revealed great numbers of larvae during the months of July in the waters over both regions from which the substratum samples were obtained.

The two substrata differed markedly. That taken from the area where juvenile snails were abundant was composed largely of coarse sand and fine gravel. It was poorly sorted, with a median grain size of 0.9 mm. An abundance of living diatoms was present in this substratum. The other substratum was rich black and probably somewhat anaerobic since a trace of hydrogen sulfide odor could be detected. This material was less than 0.06 mm. in median diameter and may be characterized as silt. Viewed under the microscope it appeared to contain large amounts of organic detritus. The mechanical analyses of the substrata are summarized in Table II. The substrata used in the experiments were not screened, but all larger organisms were removed from them with forceps under a dissecting microscope.

TABLE III

The preference of Nassarius obsoletus *for a favorable substratum for metamorphosis (results from seven experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Favorable substratum (#1, Table II)	0.0	84.4 ± 8.3
Unfavorable substratum (#3, Table II)	0.0	51.6 ± 7.6
Without substratum	0.0	30.2 ± 11.0

The percentage metamorphosis on the two substrata may be compared in Table III. In the coarse substratum 84.4% of the larvae metamorphosed, whereas on the fine black organic substratum 51.6% ended their planktonic existence. This difference is statistically significant (P equals less than 0.025). The difference between the fine black substratum and the control without substratum was not significant (P equals 0.20). The mean age of the larvae at the beginning of the experiment was 25.0 days.

The experiments show that substrata favored in the laboratory are similar to those preferred under natural conditions, and that there is a correspondence between experimental results and field observations. The general application of these results should be viewed with considerable reservation, however, pending further experiments using other favorable and unfavorable bottom sediments.

PROPERTIES OF THE SUBSTRATUM WHICH INDUCE METAMORPHOSIS

Numerous experiments indicate that the induction of metamorphosis by a favorable substratum is not a direct function of its physical character, but rather the result of its biological and chemical properties. Two lines of evidence support this conclusion.

a. *Physical properties of the substratum*

During the course of many experiments, sediments have been used from widely diverse regions. Mean grain size and sorting have differed greatly. Of those

substrata that have been tested experimentally and proven effective in inducing metamorphosis, the greatest extremes in physical properties are to be found in substrata #1 and #2 in Table II. The former, from the head of Great Pond, East Falmouth, Massachusetts, is poorly sorted gravel and sand. The latter, from Barnstable Harbor, Cape Cod, Massachusetts, is well sorted and consists primarily of fine reworked glacial sand. For the purpose of the experiments, the Barnstable Harbor substratum was passed through a Number 2 (316 μ) bolting cloth. This sieving had little effect on the physical character of the sediment, removing at most perhaps 2% of the total sample, *i.e.*, that portion exceeding 0.3 mm., in diameter. The Great Pond sample was not screened, but mollusks and annelids were removed. Table IV shows the results obtained with these substrata: in the Great Pond sample 84.4% of the larvae metamorphosed, while in the Barnstable substrate 88.7% completed their planktonic existence. Although experiments with the Barnstable and Great Pond sediments were conducted at different times and with larvae from different cultures, it seems justifiable in this instance to compare results and to conclude that the wide difference in physical character of the substrata had no

TABLE IV

The effect of two substrata, differing widely in physical properties, on the percentage metamorphosis of Nassarius obsoletus

Substratum used	Character of substratum	No. of expts.	Mean per cent metamorphosed with substratum	Mean per cent metamorphosed without substratum
Great Pond, Falmouth, Mass. #1, Table II	0.9 mm. median diameter, poorly sorted sand and gravel	7	84.4 ± 8.3	30.2 ± 11.0
Barnstable Harbor, Mass. #2, Table II	0.1 mm. median diameter, well sorted fine sand	18	88.7 ± 3.3	21.0 ± 4.8

effect on their ability to influence metamorphosis. The experimental results are completely in accord with the observations in nature that the larvae in fact do metamorphose in large numbers on these two substrata.

It may be concluded from this evidence that, within wide limits, mean particle size and sorting have no direct effect on metamorphosis. There are, however, certain indirect effects of particle size on the biological properties of the substratum as a consequence of decreased surface with increasing particle size. ZoBell (1946) has shown, for example, that the size of bacteria populations may be related to particle size.

b. *Biological and chemical properties of the substratum*

Response of larvae to the biological and chemical attributes of the substratum was tested in a series of experiments. It was intended to alter the sediment biologically and chemically without changing its physical properties.

Treating a substratum sample by (1) incinerating, (2) oven-heating, (3) washing, (4) heating in a water bath, or (5) irradiating with ultraviolet light results

in each case in a significant reduction of the metamorphosis-inducing properties of the substratum.

Incineration was accomplished by first washing and boiling the sediment in distilled water several times to remove most of the organic material, and then placing the cleaned sediment in a crucible and heating it above the high flame of a bunsen burner for one hour. The result of this treatment was to render the substratum abiotic and to free it of all traces of organic matter.

Oven-heating the sediment was done at 210° C. for 15 to 30 hours. The dried sample when removed from the oven had a dark brown appearance and a slightly pungent odor. This substratum also was abiotic, but differed from the incinerated substratum in probably having some organic material still present.

Washing the sediment consisted of boiling it in sea water and then in distilled water for several minutes, stirring vigorously, and finally decanting the surplus fine organic material. This process was repeated until the sample appeared essentially clean, *i.e.*, until it assumed a more or less light gray color and lacked any apparent suspended material when stirred. This treatment resulted in removal of part of the organic material and perhaps much of the natural microflora.

Heating the sediment was done by placing a small flask containing some sea water and the sediment sample into a beaker of water and raising the temperature to 70° to 90° C. for five to fifteen minutes. When the experiments were performed, the sea water heated inside the flask with the substratum was used along with new sea water added to make up a volume of one-half liter. Hence no part of the original substratum could have been lost, even if some material is assumed to have gone into solution as a result of the treatment. It was hoped that as a result of this treatment most of the micro-organisms would be killed without substantially affecting the organic material in the sediment. Actually this manipulation resulted, after a period of 24 hours, in a greater total number of micro-organisms than found in the natural substratum, presumably owing to the increased growth of saprophytic bacteria on dead micro-benthos. The difference amounted to two orders of magnitude as determined by plate counts, using ZoBell's marine medium No. 2216 (ZoBell, 1946). The resulting micro-flora was also qualitatively different. The treatment did not appear to cause anaerobic conditions and no fouling of water occurred.

Irradiation with ultra-violet light was done by placing, under a 15-watt germicidal lamp, containers with sediment covered by sea water to a depth of 1½ cm. The treatment was continued 18 hours, the containers being occasionally agitated. After treatment additional sea water was added to each container. Preliminary studies showed that UV treatment reduced the number of bacteria culturable in a marine medium (ZoBell's 2216) by two orders of magnitude if plated after two hours of treatment. The results of the UV treatment were not entirely successful in that complete sterilization is not possible. There is a minimum bacterial count below which further treatment is ineffective even with frequent agitation. Moreover, subsequent to irradiation, growth of saprophytic bacteria is probably very great because of the increase in decomposable material (dead micro-benthos).

The five treatments, as is shown in Table V, all resulted in a significant reduction in the attractiveness of the substrata, as determined by their effectiveness in promoting metamorphosis. Comparison of the experimental groups with their

TABLE V

The effect of various treatments on the metamorphosis-inducing property of the substratum

Treatment of substratum	No. of expts.	Mean age of larvae at start	Mean per cent metamorphosis in treated substratum	Mean per cent metamorphosis in control (untreated substratum)	Difference between treated substratum and control
Incineration	14	24.6	7.2 ± 1.5	71.2 ± 5.3	64.0
Oven-heating (210° C.)	11	26.8	19.0 ± 4.5	69.7 ± 5.0	50.7
Washing	10	22.8	4.6 ± 1.2	51.5 ± 3.9	46.9
Heating in water bath (70°–90° C.)	24	28.4	40.0 ± 4.7	81.2 ± 3.8	41.2
UV irradiation (18 hrs.)	5	28.0	70.4 ± 6.7	91.4 ± 3.4	21.0

untreated controls gives values of P less than 0.001 in all cases except that of the UV irradiation, in which a value of 0.05 was obtained.

Inasmuch as all the treatments shown in Table V have in common the fact that they drastically affected the biological properties and to varying degrees the chemical properties of the substrata, without greatly affecting the physical properties, one may conclude that biological and chemical attributes of the substrata are responsible for their metamorphosis-inducing property.

The various treatments of the substrata in these experiments (Table V) cannot be directly compared with one another because the experiments were conducted with different samples of sediment and with larvae of different ages from different cultures. Nevertheless, the differences between the treated and the control substrata (right-hand column) give some suggestion as to the relative effectiveness of each treatment in destroying the metamorphosis-inducing property of the substrata.

Further experiments have confirmed that a statistically significant difference does indeed exist between the various treatments of the substrata and their attractiveness in inducing metamorphosis. The experiments shown in Table VI were performed with larvae from the same culture and with substratum from a single source. Their only differences were the experimental conditions indicated in the left-hand column. Heating of the substratum in a water bath to 90° C. for

TABLE VI

Relative effect of two treatments of the substratum on its metamorphosis-inducing property (results from fourteen experiments)

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Untreated substratum	1.5	71.2 ± 5.3
Substratum heated in water bath to 90° C.	3.4	33.3 ± 4.8
Incinerated substratum	0.0	7.2 ± 1.5
Without substratum	1.6	13.9 ± 2.6

15 minutes rendered it very significantly less attractive than the normal substratum; however, it also remained very significantly more attractive than incinerated substratum or the controls without substratum (P equals less than 0.001 in both instances). No significant difference resulted between incinerated substratum and the controls without substratum (P equals approximately 0.10).

These experiments show that the treatment of heating the substratum in a water bath at 90° C. yielded results which fall approximately midway between those using the natural substratum and the incinerated substratum.

The experiments on the effects of various treatments of the substratum indicated that: (1) complete removal of the micro-flora and -fauna and of all organic material rendered the substratum unsuitable for stimulating metamorphosis of veliger larvae; (2) qualitative and quantitative changes in the bacterial flora, accompanied by (a) the removal of living microscopic algae and micro-fauna and (b) the alterations in the organic material, owing to heat and addition of decomposition products from the micro-benthos, made the substratum less attractive in inducing metamorphosis; (3) the biological and possibly the chemical character of the substratum was the determining factor in making the substratum suitable for metamorphosis.

A micro-benthic community cannot be separated from the organic material in a sediment. Therefore there is no experimental procedure by which to measure the effect of a micro-benthic community as a whole upon the metamorphosis-inducing property of the substratum. Single species isolated from the substratum may be tested if members of the micro-benthos can be isolated. Preliminary experiments on the effects of benthic marine bacteria isolated from favorable substrata have not yielded consistent results. The effect of the organic material on the suitability of the substratum theoretically may be tested, but no satisfactory method is available to sterilize a substratum without altering the organic material present.

Nature of the Metamorphosis Response: Experimental Evidence for the Presence of a Soluble, Biologically Active, Substratum Factor

How do the larvae perceive a favorable substratum? Is actual contact with the substratum necessary or can the larvae distinguish a favorable place for metamorphosis by some sensory clue received from material liberated into the water? Is some biologically active substance or perhaps ectocrine (see Lucas, 1955) responsible for the response to the substratum? The experiments which follow were designed to answer these questions.

a. *Transfer of the metamorphosis-inducing factor from the substratum to adjacent water*

When sea water is permitted to stand over a substratum favorable for inducing metamorphosis of veliger larvae, it soon acquires some of the metamorphosis-inducing property. Evidently some of the qualities of the substratum are transferred to the sea water.

The water was conditioned by filling a large fingerbowl to 2 cm. in depth with a sediment favorable for metamorphosis. The remainder of the bowl was then filled with fresh, filtered sea water and allowed to stand in a south window for

several days to a week. Carefully the sea water was then siphoned off, particular care being taken to exclude particles of the sediment. Filtered sea water not treated with substratum was used as control. A total of ten different substratum samples was used. In some cases, because of the time involved in treatment, the conditioned sea water was re-used. The results of the experiments are shown in Table VII.

The mean percentage metamorphosis in the substrate-conditioned sea water was 48.5 and differed significantly from the untreated control in which only 11.2% of the larvae metamorphosed (P equals less than 0.001).

The results showed that a metamorphosis-inducing factor could be transferred from the substratum into the water to a significant degree, and that larvae were

TABLE VII

The effect of sea water conditioned with substratum on the percentage metamorphosis of Nassarius obsoletus *(results from twenty-four experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Substrate-conditioned sea water	1.1	48.5 ± 4.9
Untreated sea water control	1.2	11.2 ± 2.5

able to discriminate between water recently exposed to a favorable substratum and water not recently in contact with such a substratum.

b. *Filterability of the substrate factor through membrane filters*

The metamorphosis-inducing substrate factor, if soluble, should pass through a molecular filter unaltered. An experiment was performed similar to those just described except that the substrate-conditioned sea water was divided into two equal parts. One half was filtered through a molecular filter[4] with a pore size of 0.8 μ; the other half was retained as a control. Both water samples were then tested for their metamorphosis-inducing property against a sample of untreated sea water. The resulting metamorphosis of larvae in the unfiltered, substrate-conditioned sea water and the filtered, substrate-conditioned sea water is shown in Table VIII. The mean age of the larvae at the beginning of the experiments was 28.8 days. Twelve and three-tenths per cent of the larvae in the untreated, 46.9% of the larvae in the substrate-conditioned, and 33.0% of the larvae in the filtered, substrate-conditioned sea water metamorphosed within a period of 48 hours. The difference in results between the filtered, substrate-conditioned water and the untreated sea water was significantly different (P equal to less than 0.001). The difference between the filtered, substrate-conditioned sea water and unfiltered, substrate-conditioned sea water was not significant since the value of P was approximately 0.10.

The experiments showed that the metamorphosis-inducing property of the substratum was at least in part a water-soluble substance; previous evidence has already indicated that it must be relatively stable at temperatures likely to be encountered in nature. One may speculate that the metamorphosis-inducing factor can be adsorbed by the sediment since experiments using the substratum showed

[4] Millipore Filter Corporation, Watertown, Massachusetts.

a consistently higher per cent metamorphosis over those with substrate-conditioned sea water. Such a comparison is not valid, however, without further experiment using similar larvae and sediments. The difference between the mean percentage metamorphosis in the filtered and unfiltered substratum-conditioned sea water, though not statistically significant, may perhaps indicate that under certain circumstances some adsorption may occur either on fine suspended or colloidal particles, which are removed on filtration, or on the filter itself.

c. *Implications of the experiments using substrate-conditioned sea water*

The two series of experiments using substrate-conditioned sea water supply the evidence which makes possible a reasonable hypothesis to explain the nature of the response to the substratum. The experiments suggest that the metamorphosis response of *Nassarius obsoletus* larvae is due to a soluble substance, which is relatively stable, and of biological origin. Such a substance may justifiably be termed a "biologically active" substance. Apparently the larvae perceive this substance without contact with the substratum. The latter conclusion presupposes that the veligers must have chemoreceptors sensitive enough to discriminate between the presence or absence of the metamorphosis-inducing factor in the water.

TABLE VIII

The effect of filtrating the metamorphosis-inducing property of the substratum through a molecular filter (results from eighteen experiments)

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Substrate-conditioned sea water	1.2	46.9 ± 6.5
Molecular filtered substrate-conditioned sea water (0.8 μ pore diameter)	0.3	33.0 ± 5.6
Untreated sea water	0.3	12.3 ± 3.0

Observations on adult *N. obsoletus* under both laboratory and field conditions indicate a high degree of chemoreception. For example, snails placed in a tank with a small piece of clam meat are able to locate such food very rapidly by testing water through their siphons. Chemical sensitivity has been demonstrated on two European species of the same genus (Henschel, 1932; Weber, 1924). In view of these well-developed chemical sense organs observed in the adults of the genus, it is not too surprising to encounter a well-developed chemoreception in the creeping-swimming stage of *N. obsoletus*.

Two types of chemoreception may be distinguished (Carthy, 1958): a contact chemical sense and an olfactory sense. The distinction is largely that of the concentration required to stimulate. Inasmuch as contact with the substratum by *N. obsoletus* is unnecessary, olfaction, the more sensitive chemical sense, is probably involved in the initial response.

Once the creeping-swimming larva descends to the bottom, further testing of the substratum is possible by contact chemoreceptors. This may provide an explanation for the relatively higher percentage of metamorphosis in those experiments using a substratum over those in which substrate-conditioned water was used.

Biologically active substances encompass a wide variety of materials and have been demonstrated to be of importance in a great diversity of biological activities. The subject has recently been extensively reviewed by Collier (1953) and Lucas (1955). We shall therefore not attempt an extensive summary here. It will suffice to say that the regulation of many marine animal relationships, including commensalism, antibiosis, and predation, have been explained by the demonstration of a biologically active substance.

A large diversity of organic compounds are apparently to be found in the ocean. Collier in 1953 reviewed the types of organic substances which had been found up to that time. Among these he listed N-ethylcarbazol substances ("carbohydrates"), tryosine, tryptophane, rhamnoside, ascorbic acid, and certain other unknown materials, among these a yellow lipoid substance. To these may now be added many other materials which have recently been discovered such as B_{12}-active substances (Burkholder and Burkholder, 1956; Starr *et al.*, 1957), organic acids (Koyama and Thompson, 1959; Slowey *et al.*, 1959), and various algal (Proctor, 1957) and bacterial (Sieburth, 1959) antibiotics.

Clearly, biologically active substances must encompass a wide variety of materials. Collier (1953) believes that organic compounds may originate (1) as degradation products of dead or dying animals and plants, (2) as excretory products, (3) as diffusible metabolites, and (4) from microbial activity. In the case of the metamorphosis response of *Nassarius obsoletus* to bottom sediment we do not yet know the chemical identity nor the exact source of the biologically active material. "A mud or soil with an abundant and varied microflora should contain very nearly the gamut of microbial metabolites . . ." (Hutner and Provasoli, 1951). Moreover, it is not apparent whether more than one substance may be involved in the response of the larvae. The evidence shows no discrete threshold at which metamorphosis occurs, inasmuch as the response of a group of experimental larvae to the metamorphosis-inducing factor is not "all or none." Hence not all creeping-swimming larvae metamorphose after 24 hours, despite the presence of a suitable substratum. Obviously the metamorphosis response to the substratum is not a simple one, nor should simplicity in behavior be expected from larvae, inasmuch as they are in most anatomical and physiological respects fully as complex as adults.

Function and Ecological Significance of the Substratum in Inducing Metamorphosis in Nassarius obsoletus and Nassarius vibex

a. *Adaptive value of the response of larvae to the substratum*

Nassarius obsoletus, which ingests large quantities of bottom material by means of its proboscis, is a deposit feeder. The evidence for this comes from the examination of fecal material and digestive tract contents. Since a crystalline style is present (Jenner, 1956), it is presumed that large amounts of carbohydrates are digested from the microflora within the sediment. Living bivalves of any size or species which have been tried in the laboratory are consistently refused, including those which are frequently associated with *N. obsoletus* (*e.g.*, recently metamorphosed *Venus mercenaria* and juvenile *Gemma gemma*). Subsistence from dead mollusks or Crustacea is possible; indeed, on intertidal flats one frequently encounters aggre-

gations of several hundred *N. obsoletus* feeding on some dead marine organism. Such dead organisms cannot, however, form a principal item of diet because of the large size of the *N. obsoletus* populations found on the intertidal flats.

Observations on another species of the genus *Nassarius* suggest that there is a correspondence between the metamorphosis response to bottom sediment and the adaptive value of such a response. *Nassarius vibex*, which is sometimes found sympatrically with *N. obsoletus*, is a scavenger. It is found much less abundantly than *N. obsoletus*, as would be expected by its trophic position in the community in which it lives. Experimentally we have shown that *N. vibex* does not metamorphose in response to a bottom sediment. A series of eleven experiments resulted in $67.4 \pm 11.8\%$ metamorphosis when sediment was present on the bottom of the experimental containers, whereas $62.9 \pm 11.5\%$ metamorphosed without the presence of sediment. Hence the complex behavior pattern of *N. obsoletus*, which results in the metamorphosis of larvae on a favorable substratum, has considerable adaptive value; such a response seemingly would be of no marked advantage to *N. vibex*.

b. *Ecological significance of the response of* Nassarius obsoletus *to the substratum*

"The continued presence of an animal in an environment depends upon the developmental stage in which it has the least adaptability and this stage, the weakest link of the chain, will normally be found during the breeding period and larval development." (Thorson, 1957, p. 472.) Hence, less stability should be expected of populations of organisms which have long pelagic larval stages. A comparison of arctic bottom communities, where almost all benthic organisms have direct development, with tropical benthic communities, where 75 to 80% of all bottom forms have pelagic larvae, displays no marked difference in the stability of the two (Thorson, 1957). There must exist a means, aside from chance, by which tropical benthic communities maintain their relative stability. Indeed the stability of any bottom community which depends largely on recruitment of pelagic larvae needs explanation.

One interpretation of the relative stability of large benthic populations may be found in the response of larvae to a metamorphosis-inducing factor in the substratum or, more accurately, to a very complex behavior pattern which consists of (1) recognition of a favorable substratum or environment and (2) a response to this favorable substratum, terminating in metamorphosis. This type of response may be expected in benthic forms which are deposit feeders. Our experiments have shown that under optimum laboratory conditions larvae of *N. obsoletus* may metamorphose as early as 17 days after their emergence from the egg capsule, while in the absence of a favorable substratum the termination of planktonic existence may be delayed for as long as 20 days after the creeping-swimming stage has been attained. Although the favorable substratum is come upon accidentally, the larvae may delay their metamorphosis over a period of sufficient time so as to make such an encounter very highly probable. Estuaries, which form the chief habitat for *N. obsoletus*, often have circulations of the type which will carry swimming larvae over long distances upstream along the bottom (Pritchard, 1951). Larvae as they are carried by bottom currents over a particularly suitable region will metamorphose.

SUMMARY

1. Experimental evidence shows that the presence of a favorable substratum has a marked effect on the metamorphosis of *Nassarius obsoletus* larvae. In the presence of a natural substratum the percentage metamorphosis was very significantly greater than in controls without such natural substratum. Larvae postponed metamorphosis for over two weeks when a desirable substratum was not encountered.

2. Certain substrata were preferred over others; a significant difference in the response of *N. obsoletus* larvae to different sediments was demonstrated experimentally. A correspondence exists between substrata preferred under natural conditions and those favored in experiments in the laboratory.

3. The physical properties of a substratum, such as median grain size and sorting, do not directly influence the metamorphosis of *N. obsoletus* veliger larvae. This was demonstrated both experimentally and by field observations.

4. Certain biological properties are important in making the substratum attractive for the metamorphosis of *N. obsoletus* veligers. This was shown by the differences in settlement obtained experimentally when the sediment was treated in various ways. When a sediment was rendered completely abiotic by incineration, no significant difference existed between the percentage metamorphosis on such a substratum and in controls without substratum. If the biological characteristics of the sediment were drastically altered by heating in sea water, the attractiveness of the substratum to metamorphosis by veliger larvae was significantly reduced, but it remained nevertheless significantly more attractive for metamorphosis than a substratum which had been made completely abiotic.

5. The metamorphosis-inducing properties of a substratum may be transferred to the adjacent water, as was demonstrated experimentally.

6. Experimental evidence showed that the metamorphosis-inducing factor from the substratum is probably a water-soluble substance.

7. Inasmuch as the response to a metamorphosis-inducing factor is possible without contact with the substratum, a rather sensitive chemoreception by *N. obsoletus* larvae must be involved. Stimulation may be followed by a complex behavior pattern which includes further testing of the substratum and subsequent metamorphosis.

8 Since *N. obsoletus* is primarily a deposit feeder, the selection of a favorable substratum has considerable adaptive value.

9. The ability to delay metamorphosis and the response to a favorable substratum greatly increase the probability that the larvae of *N. obsoletus* will terminate their pelagic existence in a favorable habitat.

LITERATURE CITED

AYERS, J. C., 1959. The hydrography of Barnstable Harbor, Massachusetts. *Limnol. Oceanogr.*, **4**: 448–462.

BURKHOLDER, P. R., AND L. M. BURKHOLDER, 1956. Vitamin B_{12} in suspended solids and marsh muds collected along the coast of Georgia. *Limnol. Oceanogr.*, **1**: 202–208.

CARTHY, J. D., 1958. An Introduction to the Behavior of Invertebrates. George Allen and Unwin, Ltd., London. xiv + 380 pp.

COLLIER, A., 1953. On the significance of organic compounds in sea water. *Proc. Eighteenth N. A. Wildlife Conf.*, pp. 463–468.

DAY, J. H., AND D. P. WILSON, 1934. On the relation of the substratum to the metamorphosis of *Scolecolepis fuliginosa* (Claparède). *J. Mar. Biol. Assoc.*, **19**: 655–662.

HARRINGTON, C. R., 1921. A note on the physiology of the ship-worm (*Teredo norvegica*). *Biochem. J.*, **15**: 736–741.

HENSCHEL, J., 1932. Untersuchungen über den chemischen Sinn von *Nassarius reticulata*. *Wiss. Meeresunters., Abtl. Kiel*, **21**: 133–158.

HULBURT, E. M., 1956. The phytoplankton of Great Pond, Massachusetts. *Biol. Bull.*, **110**: 157–168.

HUTNER, S. H., AND L. PROVASOLI, 1951. The phytoflagellates. pp. 27–128. *In:* Biochemistry and Physiology of Protozoa, Vol. I, ed. by A. Lwoff. Academic Press, New York.

JÄGERSTEN, G., 1940. Die Abhängigkeit der Metamorphose vom Substrat des Biotops bei Protodrilus. *Arkiv f. Zool.*, **32A** (No. 17): 1–12.

JENNER, C. E., 1956. The occurrence of a crystalline style in the marine snail, *Nassarius obsoletus*. *Biol. Bull.*, **111**: 304.

KOYAMA, T., AND T. G. THOMPSON, 1959. Organic acids in sea water. *Preprints Internat. Oceanogr. Congr.* 1959 [New York], AAAS, Washington, D. C., p. 925.

LUCAS, C. E., 1955. External metabolites in the sea. Pap. Mar. Biol. and Oceanogr., *Deep-Sea Res.*, Suppl. to Vol. **3**: 139–148.

MORTENSEN, TH., 1921. Studies of the Development and Larval Forms of Echinoderms. Gad, København (Copenhagen). 266 pp.

MORTENSEN, TH., 1938. Contributions to the study of the development and larval forms of Echinoderms. IV Kgl. *Danske Vidensk. Selsk. Skrifter, Naturv. og Math. Afd. Rackke* 9, **7**: 1–59.

NYHOLM, K., 1950. Contributions to the life-history of the Ampharetid, *Melinna cristata*. *Zool. Bid. Uppsala*, **29**: 79–91.

PRITCHARD, D. W., 1951. The physical hydrography of estuaries and some applications to biological problems. *Proc. Sixteenth N. A. Wildlife Conf.*, pp. 368–376.

PROCTOR, V. W., 1957. Studies of algal antibiosis using *Hematococcus* and *Chlamydomonas*. *Limnol. Occanogr.*, **2**: 125–139.

SCHELTEMA, R. S., 1956. The effect of substrate on the length of planktonic existence in *Nassarius obsoletus*. *Biol. Bull.*, **111**: 312.

SIEBURTH, J. M., 1959. Antibacterial activity of Antarctic marine phytoplankton. *Limnol. Occanogr.*, **4**: 419–424.

SILÉN, L., 1954. Developmental biology of Phoronidae of the Gullmar Fiord area (west coast of Sweden). *Acta Zoologica*, **35**: 215–257.

SLOWEY, J. F., L. M. JEFFREY AND D. W. HOOD, 1959. Characterization of the ethyl acetate extractable organic material of sea water. *Preprints Internat. Occanogr. Congr.* 1959 [New York], AAAS, Washington, D. C., p. 935.

SMIDT, E. L. B., 1951. Animal production in the Danish Waddensea. *Medd. Komm. Danmarks Fisk. Havunders., Ser. Fiskeri*, **11**(6): 1–151.

STARR, T. J., M. E. JONES AND D. MARTINEZ, 1957. The production of vitamin B_{12}-active substances by marine bacteria. *Limnol. Occanogr.*, **2**: 114–119.

THORSON, G., 1957. Bottom communities (sublittoral or shallow shelf). pp. 461–534. *In:* Treatise on marine ecology and paleoecology, Vol. I: Ecology, ed. J. W. Hedgpeth. *Geol. Soc. Amer., Mem.* **67**. viii + 1296 pp.

WEBER, H., 1924. Ein Umdreh und ein Fluchtreflex bei *Nassa mutabilis*. *Zool. Anz.*, **60**: 261–269.

WILSON, D. P., 1932. On the mitraria larvae of *Owenia fusiformis* Delle Chiaje. *Phil. Trans. Roy. Soc. London, Ser. B*, **221**: 231–334.

WILSON, D. P., 1937. The influence of the substratum on the metamorphosis of Notomastus larvae. *J. Mar. Biol. Assoc.*, **22**: 227–243.

WILSON, D. P., 1948. The relation of the substratum to the metamorphosis of Ophelia larvae. *J. Mar. Biol. Assoc.*, **27**: 723–760.

WILSON, D. P., 1951. Larval metamorphosis and the substratum. *Ann. Biol.* (*Paris*), **27**: 259–269.

WILSON, D. P., 1952. The influence of the nature of the substratum on the metamorphosis of the larvae of marine animals, especially the larvae of *Ophelia bicornis* Savigny. *Ann. Inst. Oceanogr. (Monaco)*, **27**: 49–156.

WILSON, D. P., 1953a. The settlement of *Ophelia bicornis* Savigny larvae. The 1951 experiments. *J. Mar. Biol. Assoc.*, **31**: 413–438.

WILSON, D. P., 1953b. The settlement of *Ophelia bicornis* Savigny larvae. The 1952 experiments. *J. Mar. Biol. Assoc.*, **32**: 209–233.

WILSON, D. P., 1954. The attractive factor in the settlement of *Ophelia bicornis* Savigny. *J. Mar. Biol. Assoc.*, **33**: 361–380.

WILSON, D. P., 1955. The role of micro-organisms in the settlement of *Ophelia bicornis* Savigny. *J. Mar. Biol. Assoc.*, **34**: 513–543.

ZOBELL, C. E., 1946. Marine Microbiology. Chronica Botanica Co., Waltham, Massachusetts. 240 pp.

A PERFUSION STUDY OF THE MOVEMENT OF STRONTIUM ACROSS THE GILLS OF RAINBOW TROUT (SALMO GAIRDNERII)

R. H. SCHIFFMAN [1]

Biology Operation, Hanford Laboratories, General Electric Company, Richland, Washington

Strontium uptake by fresh-water and marine fishes has been previously studied by either feeding, immersing, or otherwise exposing the intact fish to the isotope, and counting the activity gained by the animal. These methods give an understanding of the gross over-all uptake and elimination of the isotope, but offer little or no comprehension of the function of the gills or the mechanisms involved.

Preliminary feeding and immersion studies in our laboratory indicated that the gills of the rainbow trout were a major pathway for the uptake of strontium from water, although strontium was not concentrated in the fish to any great degree. In addition, the gills of fish are known to have an important role in homeostasis by their ability to actively take up some ions and to excrete metabolic products. Therefore, it was decided to investigate the rates of influx and outflux of strontium across the gill membrane as a first step in the study of the metabolic pathways and regulation of incorporated strontium in fish.

Keys' (1931) basic method for the perfusion of the gills of the eel was adapted for rainbow trout. The results obtained by this method suggest outflux rates compatible with the relatively poor ability of fish to concentrate strontium in the body from the water or food.

Materials and Methods

A diagram of the gill perfusion preparation is presented in Figure 1. The internal system consisted of a perfusion pump to force a modified Ringer's solution through the gills, out the dorsal aorta, and into a reservoir. The pump simultaneously drew the fluid from the reservoir into a scintillation detector from which it was recirculated through the gills. The scintillation detection system consisted of a well-type crystal which contained a coil of small-bore glass tubing through which the perfusate flowed. The output of the detector was fed to a pulse height analyzer focused on the .513 mev peak of Sr^{85}. The signal from the analyzer was integrated by a precision count rate meter and recorded on a Sanborn four-channel recorder.

In addition to isotopic concentration, the flow, volume, and pressure of the internal system were recorded electronically. Flow and volume were recorded as hydrostatic pressure in the reservoir (Fig. 1, g). Efferent flow from the gills returned to this reservoir via an automatic syphon (Fig. 1, f) which emptied when

[1] Based on work performed under Contract No. AT(45-1)-1350 for the U. S. Atomic Energy Commission.

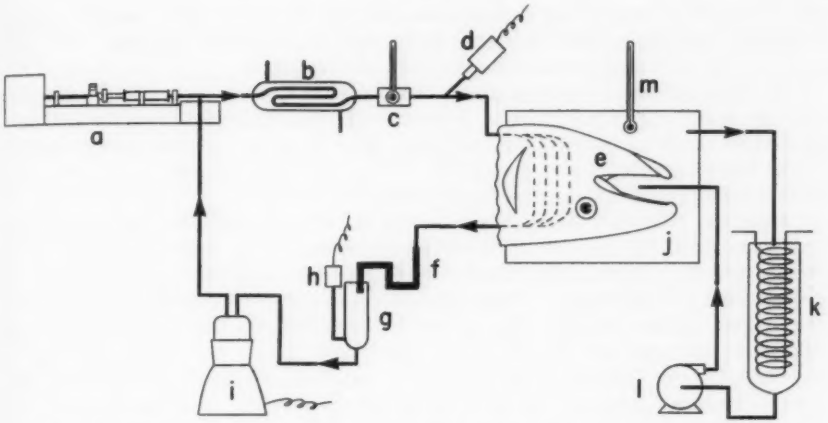


FIGURE 1. Diagram of gill perfusion preparation. Internal system: (a) perfusion pump, (b) cooling coil, (c) thermometer, (d) electronic pressure transducer, (e) fish, (f) automatic syphon, (g) reservoir with sidearm and pressure transducer (h) to measure volume and flow, (i) scintillation detector. External system: (j) diaphragm tank, (k) reservoir with cooling coil, (l) pump, (m) thermometer.

it attained its full capacity of 2.8 ml. This addition to the reservoir caused a measurable increase in hydrostatic pressure, and by use of the time scale on the electrogram, flow could be calculated. By the same means, an over-all decrease in hydrostatic pressure could be calibrated as a loss of volume in the internal system. Internal system pressure was measured as mm. Hg by a standard Sanborn medical pressure transducer (Fig. 1, d).

The Ringer's solution used had the following composition:

5.0 mEq K^+/l.
6.5 mEq Ca^{++}/l.
109 mEq Na^+/l.
112 mEq Cl^-/l.

This particular balance, which contains more Ca^{++} than frog Ringer's solution, had been found to support a trout's heart satisfactorily. Just before use, 0.4 g. $NaHCO_3$ and 0.9 g. glucose per liter of stock were added for buffer and nutrient.

The external system consisted of two liters of fluid which was pumped through the buccal and opercular cavities in order to maintain a good flow over the gills. Both the internal and external solutions were buffered in a like manner and aerated with 95% oxygen + 5% carbon dioxide gas so that the pH of the fluids remained at 6.5 to 6.7 throughout all experiments. Temperature was controlled in both systems at 13° C., an optimum for rainbow trout.

The fish was anesthetized in a solution of 0.2 g./liter of MS-222 (Tricaine Methanesulfonate, Sandoz) until respiratory movements ceased. After being weighed, the fish was placed in a holder and a median ventral incision was made from just anterior to the anus to about one centimeter posterior to the insertion

of the pectoral fins. Then heparin was injected into the heart and the gastro-intestinal tract, liver, gall bladder, gonads, and air sac were removed. All main arteries, the air bladder, and the esophagus were ligated.

A cross-sectional incision was made in the mid-kidney to open the dorsal aorta, and the heart was fully exposed. The ventricle was separated from the atria and opened to receive a polyethylene cannula. The tip of the cannula was positioned in the bulbus arteriosus and tied. The time period from the start of the operation to this point was no longer than 6 or 7 minutes.

Once the gills were being perfused with the Ringer's solution, the dorsal aorta was cannulated by exposing the aorta at the site of the earlier incision in the kidney and inserting a snug-fitting polyethylene tube until its tip lay well in the region of the head kidney. Successful positioning was obtained by palpation of the kidney as the cannula was inserted. Once positioned, slight vacuum was applied to the open end to establish a good flow. This end of the cannula was kept about 5 cm. below the level of the fish, to avoid extreme pressure in the gills. Due to vascular resistance in the healthy gills a pressure of 45 to 75 mm. Hg

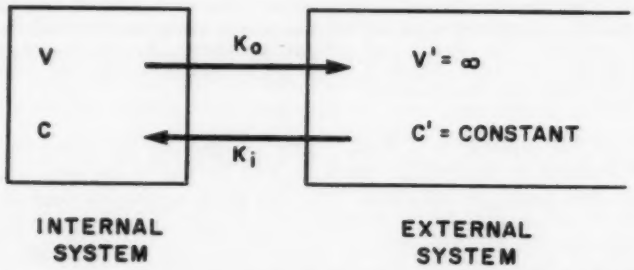


FIGURE 2. Model of two-compartment system as used in gill perfusion studies. C and C′ = concentration, V and V′ = volume.

was present in the afferent section of the system. Additional afferent pressure gained by the raising of the tip of the cannula will destroy the preparation.

Once the afferent and efferent flows were well established, the coelom was filled with a thick solution of gelatin just warm enough to be fluid. This solidified rapidly and sealed all bleeders. The fish's head was then inserted through the rubber diaphragm into the tank; the external system was filled with an appropriate fluid and circulation begun.

After the preparation had been completed and the blood flushed out of the gills, the flow from the efferent cannula was directed into the flow meter and recirculation was started.

The basic model of the preparation (Fig. 2) shows V′, the external volume, to be essentially infinite and C′, the external concentration, to be constant, due to the comparatively large volume of the external system and the short period of data collection. Actual measurements of concentrations in this system before and after experiments proved these assumptions to be valid.

Errors caused by volume changes due to osmotic forces or leakage from the internal system were difficult to evaluate. Leakage through the gills was found

to be the most serious. By minimizing the time period during which data were collected it was felt that these errors would have no serious reflection on the basic conclusions.

Keys (1951) fully discussed the difficulties encountered in this type of experimentation.

Results

Data acquired by this experimental method, using strontium-85 as a tracer ion, were considered as a linear regression of the internal concentration against time. Although the extended curve from transfer data of this type would be expected to be exponential, it was discovered that the linear treatment would introduce little error at this point and eliminate much of the difficulty encountered in endeavoring to handle both the influx and outflux with the same mathematical approach.

The equations

$$\frac{C_t \times V}{t \times A \times C'_o} = I \tag{1}$$

and

$$\frac{C_t \times V}{t \times A \times C_o} = O \tag{2}$$

were applied to the data for calculation of the influx rate (I) and outflux rate (O). C_t is the internal concentration at time t. C'_o and C_o are the initial external and internal concentrations, respectively. V is the initial internal volume, and A is the area of the gills. The area of the gills in relation to body weight was estimated from Price's (1931) figure of 2.2 cm.^{2} of gill area/kg. of body weight, which was determined on the black bass, *Micropterus dolomieu*. Although species differences probably exist, the error is constant throughout this study. I and O, the flux rates, have the dimensions of velocity, *i.e.*, cm./sec., and are similar to the permeability constant (Davson, 1951).

Table I

Influx rate (I) for strontium transport through the gill of rainbow trout

Exp. no.	Nutrient Ringer's solution internal concentration at t = o (μg. Sr^{++}/ml.)	Buffered distilled water external concentration at t = o (μg. Sr^{++}/ml.)	I (cm./sec.)
1	0	Sr^{85} (trace carrier)	1.1×10^{-6}
2	0	Sr^{85} (trace carrier)	1.3×10^{-6}
3	0	Sr^{85} (trace carrier)	2.6×10^{-6}
4	0	Sr^{85} (trace carrier)	1.0×10^{-6}
10	0	10 μg./ml.	0.5×10^{-6}
15	0	1 μg./ml.	5.2×10^{-6}
16	0	1 μg./ml.	12.0×10^{-6}
17	0	Sr^{85} (trace carrier)	7.7×10^{-6}
20	0	Sr^{85} (trace carrier)	15.0×10^{-6}
Mean ± Standard Error			$5.2 \times 10^{-6} \pm 1.8 \times 10^{-6}$

TABLE II

Outflux rate (O) for strontium through the gill of rainbow trout

Exp. no.	Nutrient Ringer's solution internal concentration at t = o (μg. Sr^{++}/ml.)	Buffered distilled water external concentration at t = 0 (μg. Sr^{++}/ml.)	O (cm./sec.)
7	100 μg./ml.	0	1.1×10^{-5}
8	10 μg./ml.	0	1.1×10^{-5}
12	1 μg./ml.	0	1.8×10^{-5}
13	1 μg./ml.	0	1.3×10^{-5}
14	1 μg./ml.	0	1.4×10^{-5}
Mean ± Standard Error			$1.3 \times 10^{-5} \pm 0.1 \times 10^{-5}$

In order to illustrate the treatment of the data received from this method, a protocol of a typical influx and a typical outflux is given. The results determined in similar experiments are listed in Tables I and II.

Determination of the influx rate

Experiment 1

Fish weight: 1094 g.

Estimated gill area: 2400 $cm.^2$

Sex: Male

Internal volume: 130 ml.

Internal medium: Nutrient Ringer's solution

External medium: Buffered distilled water plus trace amount of $Sr^{85}Cl_2$. This gave 625,000 cpm. (C') in the scintillation detector.

Figure 3 shows the data from this experiment. Internal pressure, flow rate, and volume loss were used as physiological parameters of the condition of the gills. Extreme deviations in any one of these parameters would cause the experiment to be discarded.

By using the data from Experiment 1 in Equation (1) the value of I can be calculated:

$$\frac{130 \text{ cpm./cm.}^3 \times 130 \text{ cm.}^3}{2100 \text{ sec.} \times 2400 \text{ cm.}^2 \times 312{,}000 \text{ cpm./cm.}^3} = 1.1 \times 10^{-8}$$

cpm./$cm.^2$/sec./external concentration, or 1.1×10^{-8} cm./sec.

Table I shows the values of I determined in nine similar experiments. It can readily be seen that I appears to be independent of the initial concentration of the external system. Although the flow rate through the gills may or may not affect the rates developed here, every effort was made to keep this constant and all experiments were run at flow rates of 3.5 to 4.5 ml./min.

Determination of the outflux rate

Experiment 7

Fish weight: 1598 g.

Estimated gill area: 3500 $cm.^2$

Sex: Female

Internal volume: 80 ml.

Internal medium: Nutrient Ringer's solution plus 100 μg. Sr^{++}/ml. labeled with Sr^{85}. When circulated through the detector a count rate of 72,000 cpm. was obtained, corresponding to 360 cpm./μg. Sr^{++}.

External medium: Glass-distilled water buffered with 0.4 g. $NaHCO_3$/l.

The change in the concentration of Sr^{++} in the internal medium was estimated from the count rate resulting from the Sr^{85}, and is shown in Figure 4.

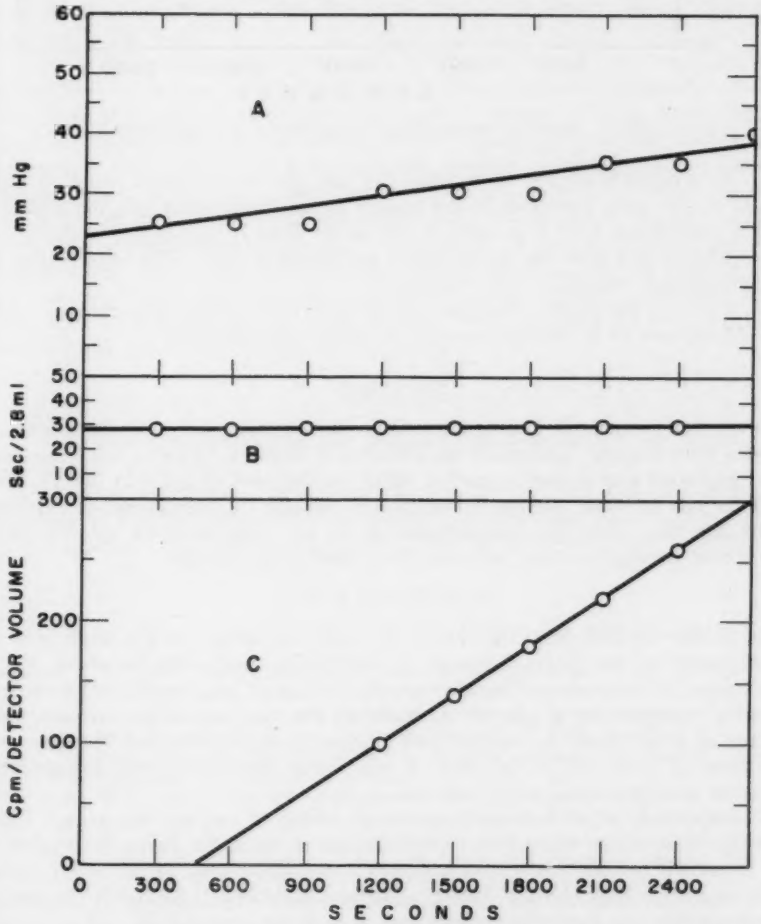


FIGURE 3. Data taken from original electrogram recorded during Experiment 1. (A) Internal pressure in mm. Hg; (B) flow in sec./2.8 ml.; (C) count rate due to Sr^{85} in cpm./detector volume. Points are discrete measurements from a continuous recording.

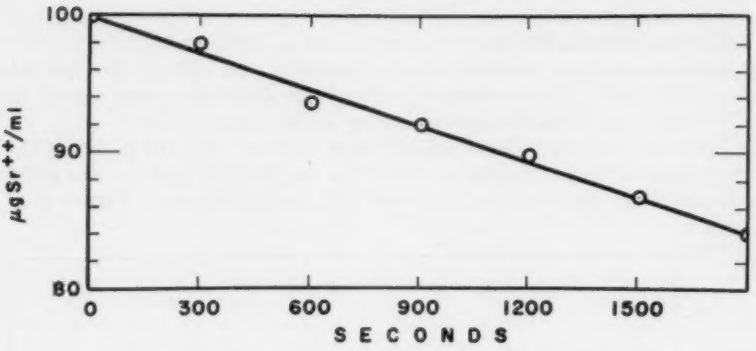


FIGURE 4. Internal concentration during course of Experiment 7.

In order to illustrate the fact that "C" can also be expressed in μg. Sr^{++}/ml., and to give some indication of the amount in μg. crossing the gill, the data from this experiment were expressed in μg. rather than in counts per minute.

The outflux rate can be calculated by Equation (2). The outflux rate for experiment 7 was 1.1×10^{-5} cm./sec.

Table II shows the O values determined in five similar experiments. Like I, O appears to be independent of the initial concentration.

DISCUSSION

Since the variance of O is greater than the variance of I, and the number of samples were unequal, Cockran's modification of Behrens-Fisher t test (Snedecor, 1956) was used and showed a marked difference between O and I at the 5% level.

If the rate at which the ion is transported through the membrane, as expressed by the flux rate, times the concentration of the ion, represents the amount that is transported through an area per unit time, then at equilibrium:

$$I\,C' = O\,C \tag{3}$$

It can readily be seen from the values of I and O obtained in this study that the concentration in the internal system at equilibrium will only be about 4% of the external concentration. In other words, Sr^{++} must be pumped out of the gills against a concentration gradient. In order to test this conclusion, an experiment was run in which both the internal and external systems contained the same concentrations of 1 μg. $Sr^{88} + Sr^{85}$/ml. It was found that the internal concentration decreased at an O of 2.2×10^{-5} cm./sec.

The apparent active transport can be explained in one of two ways. Either strontium is in direct competition with calcium in an active pump mechanism or it is a passive response to establish electrical neutrality, due to the active uptake of Na^+ and other ions (Krogh, 1939). Further study of this transport mechanism, as well as Sr^{++} vs. Ca^{++} relationships, are now being undertaken.

Since fish in fresh water tend to excrete water and conserve salts, and experiments have shown that fresh-water fish will accumulate strontium (Cook,

unpublished data; Saurov, 1957; Schiffman, 1959) from water at a ratio of approximately 1.5:1, there must be a mechanism to convert the ionized strontium to a non-diffusible form. Protein binding in the blood is just such a mechanism. Preliminary experiments in our laboratory, using both ultra-filtration of serum and dialysis of whole blood against Ringer's solution, indicate approximately 50% or more of the blood burden is in the non-dialyzable form, probably protein-bound.

Extravascular binding sites must also be taken into account since bone appears to bind strontium as tenaciously as calcium, although the rates of binding are probably much slower than those discussed here. The high outflux rate of diffusible strontium is also reflected in the relatively inferior ability of fish to concentrate strontium in the body.

A transport system for calcium, such as this postulated for strontium, would be ideal for fish such as the steelhead trout (*Salmo gairdnerii gairdnerii*) that migrate between fresh and salt water. A rate of protein binding faster than the O for Ca^{++} would allow for an uptake of Ca^{++} limited only by the availability of calcium in the environmental water, and the number of binding sites. Once the calcium content of the environmental water is raised beyond a certain degree the binding properties of the blood and extravascular sites would become saturated and the gill would become an organ of excretion of the excess diffusible portion of blood calcium. This mode of regulation would present a picture such as found by Houston (1959). He noted a definite increase in cellular calcium but a relatively stable plasma calcium in steelhead trout when adapting to sea water. Experiments are under way to test this hypothesis.

Summary

1. A perfusion technique for the study of transport across the intact gill of rainbow trout is described.

2. It was found that the outflux rate of 1.6×10^{-6} cm./sec. was greater than the influx rate of 5×10^{-8} cm./sec. for strontium, and that strontium would go out of a fish gill against a concentration gradient under these experimental conditions.

LITERATURE CITED

Davson, Hugh, 1951. A Textbook of General Physiology. The Blakiston Company, New York.

Houston, A. H., 1959. Osmoregulatory adaptation of steelhead trout (*Salmo gairdneri* Richardson) to sea water. *Canad. J. Zool.*, **37**(5): 729–748.

Keys, Ancel B., 1931. The heart-gill preparation of the eel and its perfusion for the study of a natural membrane *in situ*. *Zeitschr. vergleich. Physiol.*, **15**: 352–363.

Krogh, August, 1939. Osmotic Regulation in Aquatic Animals. Cambridge University Press, London. Pp. 130–153.

Price, J. W., 1931. Growth and gill development in the small-mouthed black bass, *Micropterus dolomieu*, Lacépède. Ohio State University Press Contribution No. 4: 1–46.

Saurov, M. M., 1957. Radioactive contamination of fish in water containing strontium. Translated from a publication of the State Publishing House of Medical Literature, Moscow. *AEC-tr-3746:* 1–12.

Schiffman, R. H., 1959. The uptake of strontium from diet and water by rainbow trout. Hanford Biology Research Annual Report 1958. *Document HW-59500:* 16–19.

Snedecor, George W., 1956. Statistical Methods. Iowa State College Press, Ames, Iowa.

REGENERATION OF THE ENTEROPNEUST, SACCOGLOSSUS KOWALEVSKII

KENYON S. TWEEDELL

University of Notre Dame, Notre Dame, Indiana, and Marine Biological Laboratory, Woods Hole, Massachusetts

Aside from the morphological similarities which they share with the chordates (Hyman, 1959), the Enteropneusta have developed regenerative methods common to both the invertebrates and the vertebrates. The regenerative capacity of the tongue worms has been noted in early reports by Spengel (1893) in *Glossobalanus minutus*, by Willey (1899) in *Ptychodera flava*, by Cori (1902) in *Balanoglossus clavigerus* and Kuwano (1902) in *Balanoglossus misakiensis*. The first thorough investigation of regeneration was done on the Mediterranean form, *Glossobalanus* (*Ptychodera*) *minutus* by Dawydoff (1902, 1907, 1909). More recently Rao (1955) has reinvestigated regeneration in *Ptychodera flava*. A form of asexual reproduction and subsequent regeneration of tail fragments in *Balanoglossus capensis* was reported by Gilchrist (1923). All of these forms are members of a highly evolved family, the Ptychoderidae (Hyman, 1959). The current form under study is *Saccoglossus* (*Dolichoglossus*) *kowalevskii*, a species common to the shores of the North Atlantic. The only previous reference to regeneration in the present genus or its family was noted by Assheton (1908) in *Saccoglossus serpenticus*, found along the coast of Scotland.

Dawydoff (1909) discovered that when the animal was cut up into many parts, regeneration occurred from the anterior end of each fragment but never from the posterior end. Secondly, regeneration resulted either as a bud formation originating from a direct proliferation of the immediate cephalic surface (epimorphosis) or from a remodeling of the anterior portion of the amputated animal (morphallaxis). More often it was a combination of these two methods.

The factors that direct this polarized regeneration are not known. Conceivably, the anterior cut surface may inhibit regeneration from a more posterior locus. Similarly, the type of structure removed or the level of amputation may control the nature of the regenerative process utilized. These queries are approached here with an investigation of the regenerative potential of *Saccoglossus kowalevskii*.

Materials and Methods

Living specimens of *Saccoglossus kowalevskii* were provided by the Supply Department at the Marine Biological Laboratory in Woods Hole, Massachusetts. Additional animals were collected by the author on the mud flats at Cotuit in West Falmouth, Massachusetts.

Both pre- and post-operative animals were maintained in the laboratory as follows. Individual animals were placed in small dishes (3×1 inches) filled

with a mixture of clean sand and a small amount of organic material. The dishes were then submerged in two-inch-deep enameled pans filled with sea water. A constant flow of fresh sea water entered the pans and flowed over the submerged dishes, which were kept at a depth of one to two inches.

(The animals were difficult to keep in the laboratory except under ideal conditions. Many of the animals reported as "not recovered" in the results died from inadequate environments. They cannot survive long on glass in either standing or running sea water, nor with an excess of organic material from their natural environs.)

Since the trunk portion of the animal is rather fragile, the animals were removed for examination by first washing away the loose sand with a gentle stream of sea water. When the casting containing the animal was exposed, it was carefully lifted out and the animal dissected from its tube.

Prior to preservation, the animals were placed in a dish of clean sea water for several hours to allow removal of the digestive contents. All mucus and adherent sand were carefully stripped from the body. Anesthesia was produced by adding 85% alcohol drop-wise to the dish until the specimens were fully relaxed. Fixation was in Stockard's solution or in 10% formalin in sea water. The animals were sectioned at 10 micra and stained with Delafield's hematoxylin and Eosin-Azure II.

General Procedure. In each series, the animals were amputated transversely, with successively larger pieces being removed in succeeding experiments, beginning at the anterior end. This provided a single amputation surface on each of two animal fragments, a posterior surface on the more cranial portion and an anterior surface on the more caudal portion. The amputated animal was then returned to its dish and periodically observed for signs of anterior regeneration. The amputated cranial portions were also studied for any indication of posterior regeneration. Representative animals were fixed daily for two weeks and then at weekly intervals.

Results

1. *Amputation through the proboscis*

The first regenerative challenge resulted from a transverse amputation of the proboscis at a point halfway back from the tip of the animal. Near the proximal end of the proboscis four coelomic pockets surround a median group of organs, the buccal diverticulum (stomochord), which is supported by a proboscis skeleton and encased by the glomerulus and heart vesicle. Since the amputation was anterior to these organs, only the epidermis, connective tissue and musculature of the proboscis were affected. (See Fig. 1, operation A.) Of 21 operated animals, 14 regenerated the lost portion of the proboscis within seven to nine days post-amputation. Examples of the amputated fragments and regenerates were fixed at one, two and three weeks. In some cases the junction of the new regenerate and the stump was clear because of a slight telescoping between the host animal and the regenerate. (See Fig. 2.) Several of the new regenerates exhibited various degrees of bifurcation at the tip. The degree of separation ranged from slight dichotomy to almost complete separation of the proboscis regenerate, as seen in Figure 3. In all of these cases, regeneration of the amputated proboscis

occurred as a local proliferation of the tissues at the cut surface (epimorphosis) and resulted in a rapid replacement of the portions removed.

Occasionally, the amputated stump regressed instead of regenerating. Regression was noted in two cases. In one specimen examined histologically after 24 days, the original proboscis had disappeared and regression had continued to the collar. The azure-colored collar epidermis had closed the opening into the branchial chamber except for a small slit. Laterally, at the junction of the collar epidermis and the purple-staining trunk epidermis, a small proboscis formed as a bud from the trunk epidermis. (See Fig. 4.) The core of the outgrowth was filled with loose muscle fibers, connective tissues and coelomocytes.

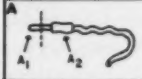
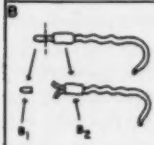
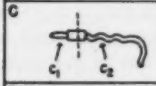
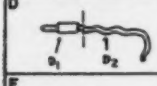
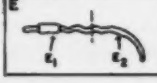
TYPE OF OPERATION	NUMBER OPERATED	ANTERIOR PIECE		POSTERIOR PIECE		REGENERATION	
		NUMBER OF:		NUMBER OF:		WITH	WITHOUT
		SURVIVORS	REGEN.	SURVIVORS	REGEN.	REGRESSION	REGRESSION
A 	21	13	0	16	16	2	14
B 	12	11	0	7	5	3	2
C 	58	48	0	9	3	2	1
D 	35	29	0	4	2?	2?	0
E 	36	32	22	27	0	0	22

FIGURE 1. Comparison of regeneration originating from the anterior or posterior pieces of transected *Saccoglossus kowalevskii* after amputation at succeeding levels.

This one example indicated that in rare instances, at least, the proboscis did not regenerate directly but rather underwent a more extensive remodeling of the existing tissues that remained in the amputated animal (morphallaxis.) The exact parameters for this type of response are not known at this time.

The amputated anterior halves of the proboscises remained alive on the surface of the sand for several weeks. These isolated fragments stayed active, moved about by ciliary action, and reacted to mechanical stimulation. This response was similar to the irritability to mechanical and photic stimulation of enteropneusts reported by Bullock (1940) and Hess (1937) in similar isolated fragments. Shortly after amputation, the proboscis fragments were able to burrow into the

sand, but later they lost this response. After three weeks, the pieces tended to decrease in size and developed rugate folds in the epidermis along the lateral surfaces. Multi-branched processes of solid epidermis often developed on the caudal surfaces of the proboscis fragments. However, in no instance was there any other evidence of regenerative activity in these isolated portions of the proboscis. This portion of the animal was apparently incapable of posterior regeneration or of reorganization into new individuals.

2. Amputation through the proboscis and sagittal splitting

In an attempt to determine the cause of the dichotomous regeneration of the proboscis, a series of 12 animals were amputated frontally through the proboscis and then the remaining stump was split sagitally to the anterior edge of the collar with iridectomy scissors. (See Fig. 1, operation B.) After five days, two animals had regressed, one to the collar region and the other to the branchial chamber. Five animals regenerated the proboscis in part. In three animals, one-half of the proboscis regenerated distally while the stump of the remaining half fused with the regenerate at the base.

Curiously, in a fourth case, the regenerated half arose from the lateral surface of the amputation stump, forming a lateral sprout from the main axis.

In two of the asymmetrical regenerates, the original organs of the proboscis had regressed. This apparently depended upon the degree of injury from the experimental incision. A new circular-shaped glomerulus, heart vesicle, and a stubby buccal diverticulum formed that extended from the proboscis stalk into the extreme proximal end of the proboscis. (See Fig. 5.) These new organs appeared to develop from the coelomocytes of the proboscis and stalk, but the exact cell precursors could not be determined from these few examples. However, each of the structures was surrounded with a very heavy non-cellular lamellated membrane, originating directly from the body of the proboscis skeleton.

In one other example, both halves of the proboscis fused and the two regenerated together. Five other animals were not recovered.

The above results suggested that the dichotomy in the previous series was the result of regeneration from two separate loci, which sometimes fused and at other times failed to do so.

3. Amputation through the collar

Each animal of this group was amputated transversely through the middle of the collar, as indicated in Figure 1, operation C. The latter half of the animal, consisting of the posterior collar region and the attached trunk, was retained, as well as the proboscis and anterior half of the collar.

The amputated animal was less capable of withstanding the operation than the anterior portion that was removed. This may have been due to the trauma of operation or possibly it was a result of mucus-collection, which had a tendency to strangle the animal unless it was removed. Out of a total of 58 operated animals, only nine amputated animals were recovered. The large number of regenerative failures from the C_2 pieces was thus, in large part, due to the very high rate of mortality among the amputees. Regression of the remaining collar was quite common and usually began within two to three days after amputation. Al-

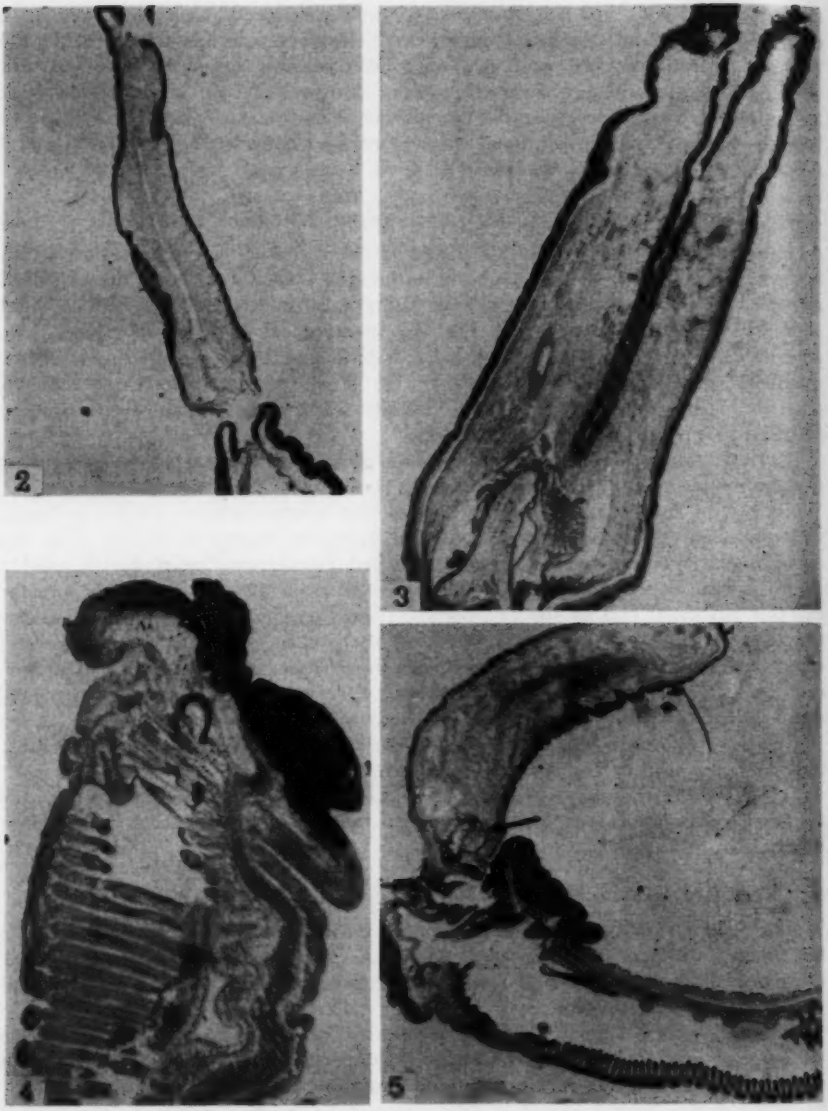


FIGURE 2. Regeneration of the distal end of the proboscis after transverse amputation halfway back from the tip. Replacement of tissue results as a direct proliferation of the amputation surface. Fixed at seven days post-amputation.

FIGURE 3. Occasional bifurcation seen in regenerates after transverse amputation of the proboscis. The buccal diverticulum, heart and glomerulus are present in the proximal portion

most all of the operated animals regressed into the branchial region of the gonadal area within five to six days post-amputation. After regression, the animals either became stabilized or degenerated completely.

Of the animals recovered, three were found in different stages of regeneration. One had completely regenerated the amputated half of the collar and a full proboscis at 26 days post-amputation. This animal had not regressed prior to regeneration. (See Fig. 6.) Two other specimens, fixed at six days post-amputation, showed initial bud or blastema formation. The latter two cases were animals which had regressed prior to the beginning of regeneration.

The isolated pieces of proboscis and attached collar showed a gradual reduction of the attached collar between five and seven days post-amputation. Collar portions were still found 14 days after amputation but at the end of 18 days, almost all of the fragments had regressed collar parts with the denuded skeleton projecting out of the proboscis. Highly differentiated structures within the proboscis or proboscis stalk, such as heart, glomerulus, and buccal diverticulum, were retained for as long as 15 days. However, these structures disappeared from the proximal end of the proboscis, leaving only the muscle and epidermal portions of the proboscis intact. Although small buds of epidermis often appeared on the posterior surface, there was no indication of posterior regeneration. These isolated proboscises remained active and survived on the surface of the sand up to four weeks, and then gradually shrank and disintegrated.

4. *Amputation posterior to the collar*

In the fourth experiment, the animals were amputated at a position posterior to the collar so that a small portion of the branchial region was included. (See Fig. 1, operation D.) Mortality here was again very high, possibly due to both mucus constriction and the inability of the trunk region to burrow. Out of 35 operated animals, only four survived. Two of these showed no sign of regeneration and had regressed to the hepatic region at the end of nine days. The remaining two had also regressed to the posterior branchial region and were fixed at seven days. Outwardly, they had developed small, yellow fleshy bulbs at the anterior end. Each bulb consisted of a median diverticulum which formed from the mucosal lining of the old branchial region. (See Fig. 7.) However, it was very doubtful that these epidermal blebs represented anything more than local metaplasia, and they were probably not true regenerates. As shown in the next section, amputation through the identical area (the post-branchial region) often produced similar small epidermal bulbs. Histological examination of the latter growths, at three weeks post-amputation, never indicated that they differentiated further.

---

of the proboscis. Regeneration here occurs as a direct proliferation from the double amputation surfaces.

FIGURE 4. Formation of a new proboscis through morphallaxis after regression into the collar region. The proboscis is forming at the junction of the collar and trunk epidermis. Fixed at 24 days post-amputation.

FIGURE 5. An asymmetrical regenerate after amputation and sagittal splitting of the proboscis stump. A new glomerulus, heart vesicle and buccal diverticulum, indicated by the arrow, are beginning to form in the proximal end of the proboscis.

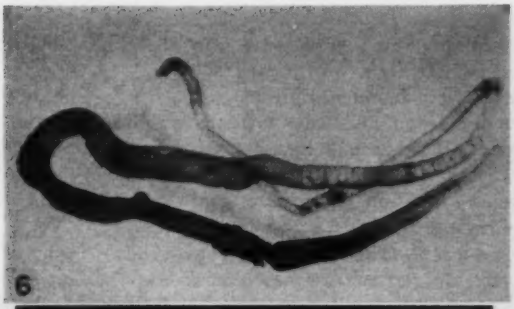


FIGURE 6. Complete regeneration of the proboscis and anterior half of the collar after amputation through the collar. This animal had not regressed prior to regeneration but had regenerated directly from existing primordia. Fixed and cleared at 26 days post-amputation.

FIGURE 7. Sagittal view of an amputated trunk which had regressed into the posterior branchial region. The arrow indicates typical epidermal outgrowths that form on the anterior surface but never differentiate further. Fixed at seven days post-amputation.

FIGURE 8. An animal which illustrates posterior regeneration originating from an intact proboscis, collar and branchio-genital region. This young, immature animal had formed a new post branchial trunk at seven days post-amputation. Arrow indicated the level of amputation.

The amputated anterior portion, consisting of proboscis, collar and a fragment of the branchial area, was self-sustaining from five to fifteen days. The newly created caudal opening remained open and, with the original mouth opening, provided double apertures into the collar region.

No proliferation was ever found on any of the anterior pieces. The branchial chamber and collar generally persisted for a week but then began to regress thereafter. Regression usually continued until only the proboscis remained. In some cases, however, the collar and trunk were still intact at the end of three and one half weeks.

5. *Amputation posterior to the branchial region*

The last and most posterior cut was made just behind the last gill opening. The point of amputation was determined by the terminal tip of the external gill

openings that taper to a point in the midline between the lateral gonads. (See Fig. 1, operation E.) Only completely intact animals were used, and both anterior and posterior pieces were kept. The anterior portion, consisting of proboscis, collar and branchio-genital region, always burrowed into the sand immediately, while the latter portion never burrowed.

At the end of three days, the anterior wound area of the posterior piece (hepatic area and caudal intestine) had healed. Of 36 operated animals, 27 pieces were still intact at the end of three days. The remainder had deteriorated. In the survivors, usually the gonadal ridges turned over to fuse with the paired, median ventral ridges, which completely sealed off the opening into the intestine. Occasionally, single or multiple transparent epidermal vesicles appeared on the healed surface, but these small blister-like growths never grew larger nor did they show further differentiation. Most of the pieces survived for seven to eight days, and at the end of one week some of these degenerated, generally starting in the hepatic region. The more anterior gonadal and posterior intestinal fragments that resulted always survived longer. In some animals, a new opening appeared in the lateral wall just behind the amputation site. Almost invariably, this was followed by an inversion of the complete trunk region behind the new opening. Thus, the entire mucosal lining was outermost while the epidermal covering now served as a lining. The posterior pieces persisted for ten days, gradually fragmenting thereafter until only long segments of the post-hepatic region remained. None of the posterior pieces had regenerated after three weeks.

For the first time in these experiments, the anterior portion, composed of proboscis, collar and branchio-genital regions, showed signs of posterior regeneration after three days post-amputation. Regeneration was first detected in the younger, immature animals but regeneration also took place in the mature animals.

As a group, the younger, immature animals had a greater growth rate than the older, mature ones. At the end of one week, each animal had developed a new post-branchial region equal in length to the intact branchio-genital region. The mean length of the regenerates at this time was 1.1 cm. (See Fig. 8.) In two animals, an entire new post-branchial trunk, equal to the animal's original length, had formed. Regional differentiation of the new trunk regenerates into gonadal, hepatic and caudal areas followed after outgrowth of the trunk. In one example, differentiation of the gonadal and hepatic regions was completed at the end of eight days.

The sexually mature animals regenerated at a slower rate than the younger forms, averaging 0.5 cm. of new growth in one week. At the end of one month, the mean length of the regenerates measured 1.6 cm. A second important difference between the immature and sexually mature animals was in the organs immediately replaced. Existing differentiated structures at the amputation surface usually accompanied the trunk outgrowths. Thus, in the short regenerates of two to three days, new gonadal tissue accompanied the regenerating trunk wall. In the females, the ovaries contained fully formed eggs, easily seen through the body wall.

Posterior regeneration was observed in 22 of 32 surviving animals. The remaining animals regressed in a manner identical to that noted in the preceding operations.

Discussion

The current experiments showed that the direction of axial regeneration varied, depending upon the level of amputation. In the anterior region of the body, regeneration never originated from a posterior amputation surface, but once the level of the branchial region was passed, regeneration was possible from a posterior amputation surface. Now, however, regeneration was not demonstrable from the anterior surfaces of lower trunk areas. These results both confirmed and differed with the results obtained by Dawydoff (1902, 1907, 1909) and Rao (1955). In Dawydoff's and Rao's experiments, the animals were generally cut into about four portions, the proboscis and collar, the branchial region, the hepatic region and the post-hepatic region. They found that anterior regeneration, where technically possible, occurred from the separate fragments, but they never reported regeneration from a posterior amputation surface in *Glossobalanus minutus* and *Ptychodera flava*. One difference in the present experiments on *Saccoglossus kowalevskii* was in the method of amputation. Each operation exposed only one cut surface on each piece, oriented either cranially or caudally. It was subsequently seen that posterior regeneration can occur from an anterior piece in the post-branchial area, a fact which diverges from other reports. It is conceivable that posteriorly-directed regeneration in the latter part of the animal was prevented by a more anterior dominant regeneration surface in the earlier experiments.

Two separate phenomena seemed related to the movement of the regeneration site posteriorly. The first, a tendency toward regression of the amputated parts, was lowest in the proboscis region. It became more common in the collar region and was found quite frequently in the trunk region. Regression always involved the loss and disappearance of structures in an orderly fashion from the wound, and should be distinguished from over-all deterioration of an animal fragment. From the present data, regression did not always lead to gradual but complete disappearance of the organism. In the first three experiments, regression was occasionally arrested by unknown parameters. Subsequently, the existing tissues were reorganized and regeneration followed.

A second observation was based on the type of regeneration involved, *i.e.*, epimorphosis or morphallaxis, and its relation to the level of amputation. When the proboscis was amputated, this almost always resulted in a quick and direct replacement of the lost portion through epimorphosis, the size of the regenerate approximating the original size of the amputated part. Transsection through the collar could result in either form of regeneration, with the rapid formation of a full-size regenerate or a small regenerate undergoing morphallaxis. When the amputation was made just behind the collar, regression always followed but regeneration was not demonstrable. In the post-branchial region, posterior regeneration took place again by the process of epimorphosis.

It appears that a certain degree of controlled regression immediately precedes regeneration whenever morphallaxis takes place. Continued regression results in no regeneration and gradual deterioration of the amputated stock. The factors that initiate and control regression are still unknown.

Summary

1. Transverse cuts, which separated the animal into two parts, were made on *Saccoglossus kowalevskii*. When transsection occurred through the proboscis or

collar region, the posterior animal fragment was capable of regenerating lost parts anterior to the amputation surface but the anterior animal portion was not able to replace more posterior parts.

2. When amputation was performed behind the branchial region, anterior parts were never formed from the posterior animal portion. However, regeneration of more posterior missing parts was now possible.

3. Regeneration occurred either from a direct proliferation of the tissues present at the amputation surface (epimorphosis) or through a remodeling of the anterior portion of the amputated fragment (morphallaxis).

4. As the locus of amputation was moved posteriorly, epimorphosis became less common and morphallaxis became the principal method of regeneration. Just behind the branchial region, successful posterior regeneration seemed to be accomplished exclusively through epimorphosis.

5. Immediately following amputation, regression often occured from the posterior fragments. If regeneration ensued, regression always preceded morphallaxis but did not precede epimorphosis.

LITERATURE CITED

ASSHETON, R., 1908. A new species of *Dolichoglossus*. *Zool. Anz.*, **33**: 517–520.

BULLOCK, T., 1940. Functional organization of the nervous system of Enteropneusta. *Biol. Bull.*, **79**: 91–113.

CORI, C. I., 1902. Über das Vorkommen des *Polygordius* und *Balanoglossus* (*Ptychodera*) im Triester Golfe. *Zool. Anz.*, **25**: 361–365.

DAWYDOFF, C., 1902. Über die Regeneration der Eichel bei den Enteropneusten. *Zool. Anz.*, **25**: 551–556.

DAWYDOFF, C., 1907. Sur la morphologie des formations cardio-péricardiques des Enteropneustes. *Zool. Anz.*, **31**: 352–362.

DAWYDOFF, C., 1909. Beobachtungen über der Regenerationsprozess bei den Enteropneusten. *Zeitschr. wiss Zool.*, **93**: 237–305.

GILCHRIST, J., 1923. Dimorphism and asexual reproduction in *Ptychodera capensis*. *J. Linnaean Soc. London*, **35**: 393–398.

HESS, W., 1937. The nervous system of *Dolichoglossus kowalevskii*. *J. Comp. Neurol.*, **68**: 161–171.

HYMAN, L., 1959. The Invertebrates: Smaller Coelomate Groups. Vol. 5, Chap. 17. McGraw-Hill, New York.

KUWANO, H., 1902. On a new enteropneust from Misaki, *Balanoglossus misakiensis* n. sp. *Annot. Zool. Jap.*, **4**: 77–84.

RAO, K., 1955. Morphogenesis during regeneration in an enteropneust. *J. Animal Morphol. Physiol.*, **1**: 1–7.

SPENGEL, J. W., 1893. Die Enteropneusten des Golfes von Neapel und der angrenzenden Meeres-Abschnitte. Fauna u. Flora d. Golfes von Neapel. 18° Monographie, p. 684, Berlin.

WILLEY, A., 1899. Enteropneusta from the South Pacific, with notes on the West Indian Species. *In:* A. Willey, Zoological Results. Part 3, Cambridge.